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Exploring the Oral Health Benefits of Levilactobacillus brevis CD2: A Translational Approach

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Abstract

Background and Aim: Emerging *in vitro* and *in vivo* evidence supports the application of probiotics as a natural approach to promoting oral and systemic health. This double-blind, randomized, placebo-controlled trial investigated the efficacy of *Levilactobacillus brevis* CD2 (CNCM I-5566), a multifunctional probiotic widely adopted in oral medicine, in preserving and enhancing key oral health parameters.

Methods: Thirty healthy adult volunteers were randomly assigned to receive four lozenges per day containing either *L. brevis* CD2 (n = 15) or a placebo (n = 15) for a duration of four weeks. Clinical assessments included full-mouth bleeding on probing (BoP) and plaque index (PI). Unstimulated saliva samples were collected to evaluate salivation rate, pH, and buffering capacity. Salivary biomarkers analysed included glucose, D-lactate, secretory immunoglobulin A (sIgA), and nitrate and nitrite levels, serving as indicators of oral nitrate-reducing capacity. The *ex vivo* nitrate-reducing potential of the probiotic lysate was also evaluated, along with its effects on pH, buffering capacity, and D-lactate levels after 3 hours of incubation. The impact of *L. brevis* on α-amylase (salivary and pancreatic) activity was also assessed. Measurements were taken at baseline, post-intervention (week 4), and two weeks after washout.

Results: After 4 weeks of supplementation, the probiotic group showed significant improvements in BoP and PI compared with baseline and placebo. Salivation rate increased significantly at both post-treatment and washout timepoints. While pH remained stable, buffering capacity improved following probiotic intake. Salivary glucose and D-lactate levels decreased, whereas sIgA levels increased and remained elevated after washout. A notable enhancement in oral nitrate-reducing capacity was observed in the probiotic group. Additionally, *L. brevis* CD2 effectively inhibited salivary and pancreatic α-amylase activity. No adverse events were reported.

Conclusions: *L. brevis* CD2 supplementation significantly improves both clinical and biochemical markers of oral health, including inflammation, plaque accumulation, salivary flow, immune response, and nitrate-reducing capacity. Its ability to modulate α-amylase activity further suggests potential systemic benefits. These results support the use of *L. brevis* CD2 as a safe and effective probiotic intervention for promoting oral and systemic health in healthy individuals.

1. INTRODUCTION

1.1. Oral Health as a Systemic Gateway

Historically, oral health has been considered a domain exclusive to dentistry, often perceived as a localized issue with limited systemic implications. However, a growing body of evidence over the past two decades has profoundly reshaped this view, positioning the oral cavity as a pivotal interface between environmental exposures and systemic physiology.

The oral microbiome, second only to the gut in terms of microbial diversity and functional complexity, is a dynamic ecosystem composed of bacteria, fungi, viruses, and archaea. In a healthy state, it contributes to mucosal homeostasis, nitric oxide (NO) metabolism, immune tolerance, and epithelial integrity. Conversely, dysbiosis within this ecosystem can lead to the proliferation of periodontopathogens, including *Porphyromonas gingivalis*, *Fusobacterium nucleatum*, and *Aggregatibacter actinomycetemcomitans*. These microorganisms can invade host tissues and enter the systemic circulation, releasing virulence factors, including lipopolysaccharides, peptidoglycans, and damage-associated molecular patterns (DAMPs). These components activate Toll-like receptors and inflammasomes, triggering systemic inflammation and contributing to endothelial dysfunction and atherogenesis [Schenkein et al, 2020; Zhu et al., 2022].

Periodontal disease (PD), a chronic inflammatory condition driven by oral dysbiosis, has emerged as a non-traditional yet modifiable risk factor for several systemic diseases, including cardiovascular disease (CVD), chronic kidney disease (CKD), diabetes mellitus, and neurodegenerative disorders. The mechanisms underlying these associations involve microbial translocation, systemic endotoxemia, immune activation, endothelial impairment, and epigenetic remodeling [Hajishengallis and Chavakis, 2021].

This growing recognition has led to the conceptualization of the oral-systemic axis, a bidirectional pathway through which oral health influences, and is influenced by, systemic conditions. The oral cavity serves not only as a reservoir for pathogenic microorganisms but also as a sentinel site for early detection of systemic imbalances.

Salivary biomarkers, for instance, reflect metabolic, immunological, and inflammatory states, offering a non-invasive window into systemic health. Moreover, the oral microbiome interacts with host physiology through metabolic pathways, such as nitrate reduction and arginine catabolism, with implications for vascular function, immune modulation, and glycemic control.

Understanding and modulating the oral-systemic axis is therefore critical for developing integrative strategies aimed at disease prevention and health promotion. In this context, microbiome-based interventions, such as targeted probiotics, are gaining attention as promising tools to preserve or restore oral homeostasis and to prevent or mitigate systemic risk.

1.2. My Personal Experience during the PhD Course

In the wake of the foregoing, during my PhD training, under the invaluable guidance of my supervisors, I had the opportunity to deepen my understanding of the human microbiome and explore how its modulation through probiotics can contribute to both oral and systemic health. Within this framework, I devoted significant effort and passion to developing a scientific review published in Trends in Cardiovascular Medicine (2023) that provides compelling evidence for the role of PD in cardiovascular pathology. The review examined the intricate relationship between PD and CVDs, identifying PD as an emerging and modifiable risk factor. PD is a chronic inflammatory condition affecting the supporting structures of the teeth, often triggered by dysbiosis of the oral microbiome. It is strongly associated with systemic inflammation, a key driver in the development and progression of CVDs. The article synthesized current evidence linking PD to specific cardiovascular conditions, including heart failure, atrial fibrillation, and atherosclerosis. It highlighted underlying mechanisms, including oral dysbiosis, inflammatory signaling, endothelial dysfunction, and epigenetic alterations. Oral dysbiosis contributes to systemic endotoxemia and chronic inflammation, thereby elevating circulating levels of inflammatory markers, including C-reactive protein (CRP), interleukin-6 (IL-6), and tumor necrosis factor-α (TNF-α), all of which are implicated in cardiovascular

pathology (**Figure 1**). Moreover, PD-associated inflammation impairs endothelial function, a precursor to vascular disease, and induces epigenetic modifications that may further influence cardiovascular risk. The review also evaluated the impact of periodontal treatment on cardiovascular outcomes. While findings remain heterogeneous, several studies suggest that non-surgical periodontal therapy can reduce systemic inflammation, improve endothelial function, and lower cardiovascular risk markers. However, limitations such as small sample sizes and short follow-up durations underscore the need for further research.

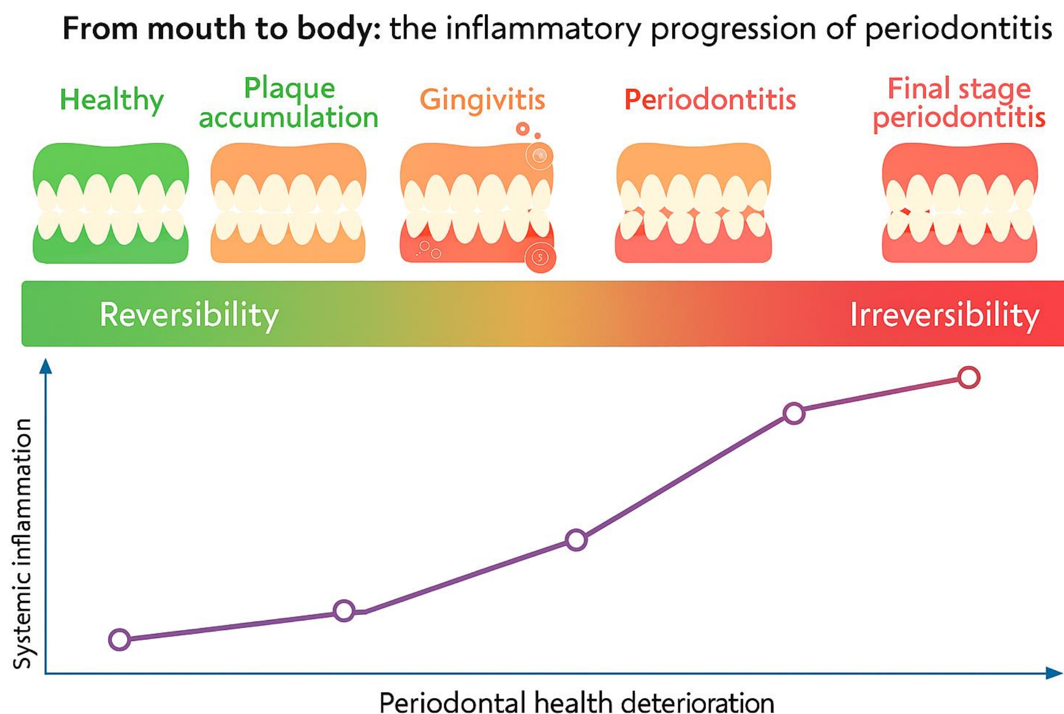


Figure 1. Natural progression of periodontitis in predisposed individuals. Biofilm accumulation causes periodontal deterioration to varying degrees depending on both personal and professional oral care habits. Gingivitis is a reversible inflammation of the gums where alveolar bone, periodontal ligament, and alveolar cement are not damaged. Its progression leads to periodontal pockets and the irreversible destruction of periodontal structures, ultimately resulting in tooth mobility and loss. Mechanistically, systemic inflammatory markers (i.e., CRP, IL-1β, IL-6, TNF-α) increase progressively with deterioration of periodontal health, with an impact on cardiovascular and general health.

In conclusion, the review strongly advocated integrating oral health care into broader health promotion strategies, emphasizing that effective management of PD may complement existing approaches to prevent or mitigate cardiovascular disease.

This experience not only enriched my scientific perspective but also reinforced the importance of interdisciplinary approaches in addressing complex health challenges.

In parallel, the review published in *Biomedicines* [Altamura et al., 2023] stressed the concept of the oral–gut–kidney axis, highlighting how oral inflammation and dysbiosis can exacerbate gut microbial imbalance, increase intestinal permeability, and promote the accumulation of gut-derived uremic toxins (GDUTs), such as indoxyl sulfate and p-cresol sulfate. These toxins are known contributors to the progression of chronic kidney disease (CKD), systemic inflammation, and cardiovascular complications [Vanholder et al., 2014; Ramezani et al., 2016]. CKD is increasingly recognized as a systemic inflammatory condition influenced not only by traditional cardiometabolic risk factors (e.g., hypertension, diabetes) but also by non-traditional contributors such as gut dysbiosis and oral disease. Our review explored the bidirectional relationships within this emerging axis, emphasizing how microbial and inflammatory crosstalk may accelerate CKD progression and its systemic sequelae.

CKD is characterized by chronic, low-grade inflammation, impaired immune responses, and elevated levels of pro-inflammatory cytokines (IL-6, TNF-α, and IL-1β), which correlate with a declining glomerular filtration rate (eGFR) and increased cardiovascular risk. Inflammasome activation, particularly NLRP3, contributes to renal fibrosis and vascular calcification, while reactive oxygen species (ROS) overproduction, driven by NOX4 and mitochondrial dysfunction, promotes endothelial injury. Dyslipidemia and altered lipid metabolism further aggravate renal and vascular damage. Moreover, the accumulation of uremic toxins and gut-derived metabolites (e.g., indoxyl sulfate, p-cresol sulfate, trimethylamine N-oxide [TMAO]) fosters systemic inflammation, immune suppression, and cardiovascular events. Hyperuricemia also activates inflammasomes and contributes to renal injury and metabolic dysfunction. CKD alters the gut microbiota composition, favoring proteolytic species and reducing the abundance of bacteria that produce short-chain fatty acids (SCFAs). Increased intestinal permeability leads to endotoxemia and

systemic immune activation. TMAO, a microbial metabolite, has been directly linked to cardiovascular mortality in CKD.

Importantly, patients with CKD exhibit increased susceptibility to periodontal disease due to uremia, xerostomia, and immune dysfunction. Periodontitis exacerbates systemic inflammation and endothelial dysfunction, potentially accelerating CKD progression. Oral dysbiosis may also influence gut microbiota, reinforcing the oral–gut–kidney axis. Interestingly, periodontal treatment has been reported to improve systemic inflammation, eGFR, and nutritional markers in CKD patients, as well as reduce cardiovascular risk and enhance erythropoietin responsiveness. Thus, the oral–gut–kidney axis represents a dynamic, clinically relevant network that influences CKD pathophysiology. Integrative strategies targeting both oral health and gut microbiota may offer novel adjunctive therapies to mitigate CKD progression and improve patient outcomes. Greater interdisciplinary collaboration between nephrologists and oral health professionals is warranted (**Figure 2**).

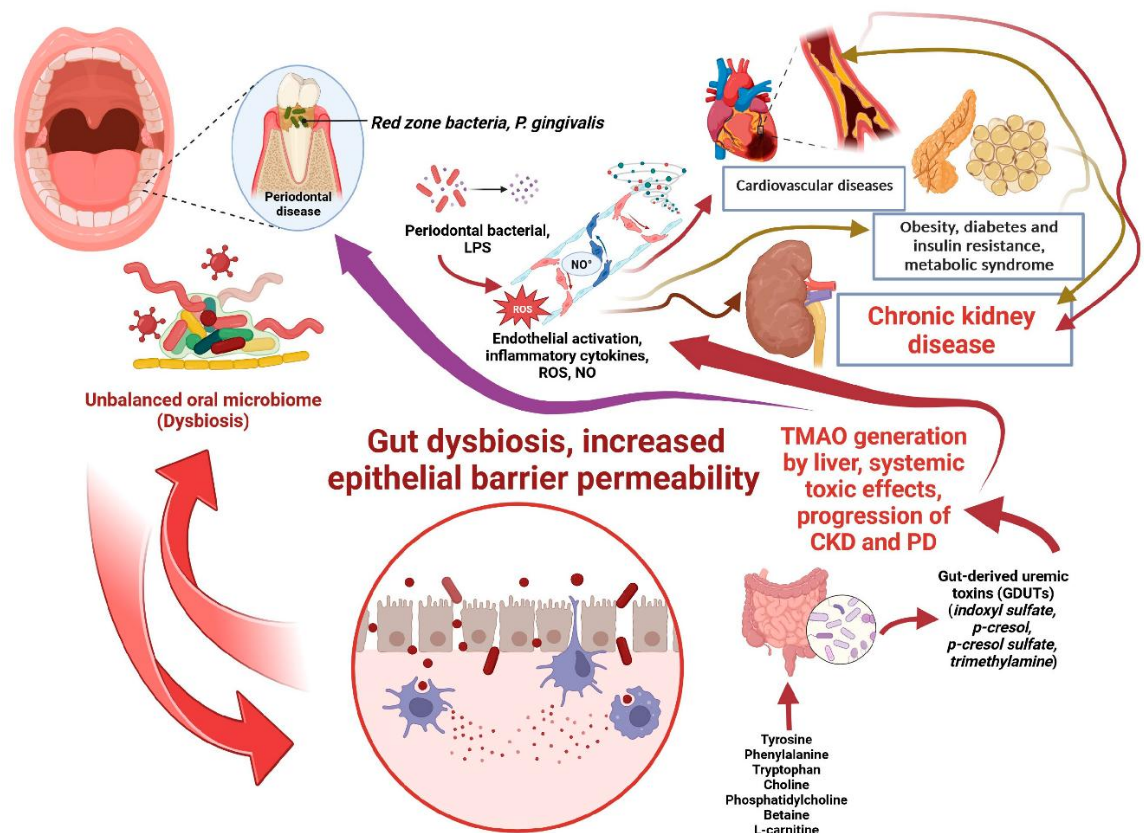


Figure 2. The oral–gut–kidney axis is a dynamic, bidirectional network in chronic kidney disease (CKD), in which oral dysbiosis and gut microbial imbalance contribute to systemic inflammation, immune dysfunction, and renal pathology. Targeted interventions, such as periodontal treatment and microbiota modulation, may mitigate CKD progression and improve clinical outcomes.

In conclusion, both review articles highlight the translational potential of probiotics in cardiovascular and renal contexts, suggesting that targeted microbial interventions may serve as effective adjunctive therapies in the management of systemic diseases associated with oral dysbiosis. Indeed, given the central role of microbial imbalance in the pathogenesis of chronic inflammatory conditions, modulation of the microbiome has emerged as a promising and innovative therapeutic strategy. This paradigm shift, from pathogen eradication to ecological restoration, opens new approaches for integrative care, where oral health becomes a cornerstone of systemic well-being.

1.3. The Oral Microbiome and Salivary Ecosystem in Health and Disease

The composition and functionality of the oral microbiome are shaped by a multitude of intrinsic and extrinsic factors, including aging, genetic predisposition, dietary habits, oral hygiene practices, pharmacological treatments (notably antibiotics), tobacco use, and systemic or immunological conditions (**Figure 3**). Perturbations in this finely balanced microbial ecosystem, commonly referred to as dysbiosis, can lead to the overgrowth of opportunistic pathogens, thereby contributing to the onset and progression of various oral diseases, including dental caries, periodontitis, and endodontic infections.

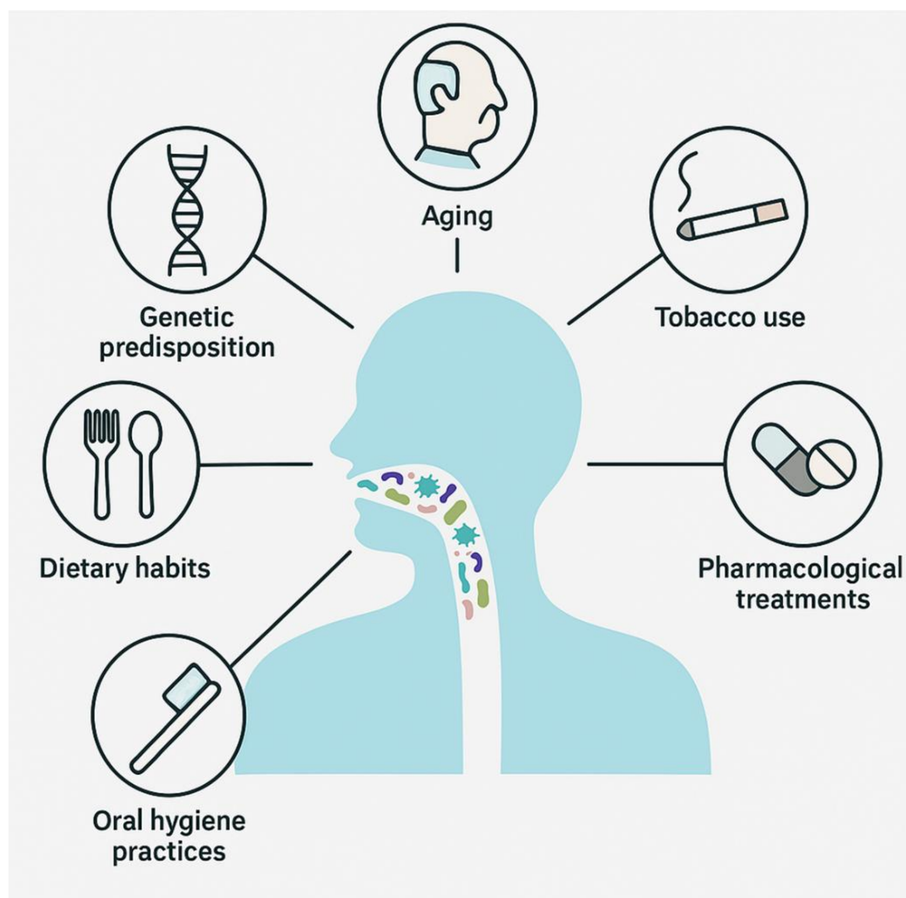


Figure 3. A multitude of intrinsic and extrinsic factors shape the composition and functionality of the oral microbiome.

Saliva plays a pivotal role in maintaining oral homeostasis [Timpel et al., 2023]. This complex biofluid has multiple functions, including aiding 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 [Dawes and Wong, 2019; Pittman et al., 2023].

1.4. Probiotics as Modulators of Oral Microbial Ecology

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 [Bowen et al., 2018; Graves et al., 2019; Menon et al., 2019; Shapiro et al., 2022; Santacroce et al., 2023]. When the balance of the oral microbiome is disrupted, it can lead to an overgrowth of potentially harmful organisms, which may contribute to the development of several oral diseases, including dental caries, periodontitis, and endodontic infections [Krishnan et al., 2016].

One emerging strategy to preserve oral health and prevent dental caries, among other oral diseases, is based on the assumption that selected probiotics can restore equilibrium in the oral microbial ecosystem [Manmontri et al., 2020]. According to the definitions provided by the United Nations Food and Agriculture Organization (FAO) and the World Health Organization (WHO), probiotics are living microorganisms that provide health benefits to the host when administered in adequate amounts [FAO/WHO, 2001]. 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 [Azad et al., 2018; Monteagudo-Mera et al., 2019; Markowiak-Kopeć and Śliżewska, 2020; Anjana and Tiwari, 2022]. Over the past two decades, scientific interest in the application of probiotics to oral medicine has grown exponentially [Yu et al., 2024]. Numerous studies have demonstrated their efficacy in managing a wide spectrum of oral conditions, including dental caries, periodontal and peri-implant

diseases, halitosis, oral candidiasis, and mucositis induced by chemoradiotherapy [Xia et al., 2021; Amato et al., 2022; Rebelo et al., 2023]. The mechanisms through which probiotics exert their beneficial effects in the oral cavity mirror those observed in the gastrointestinal tract (**Figure 4**).

These include:

- ➢ Production of short-chain fatty acids and antimicrobial peptides
- ➢ Inhibition of pathogenic biofilm formation
- ➢ Competitive adhesion to mucosal surfaces
- ➢ Coaggregation with pathogens
- ➢ Immunomodulatory and anti-inflammatory activity
- ➢ Enhancement of salivary flow and mucosal immunity.

Additionally, probiotics can co-aggregate with pathogens, exert immunomodulatory and anti-inflammatory effects, and enhance the oral microbiome [Luo et al., 2024].

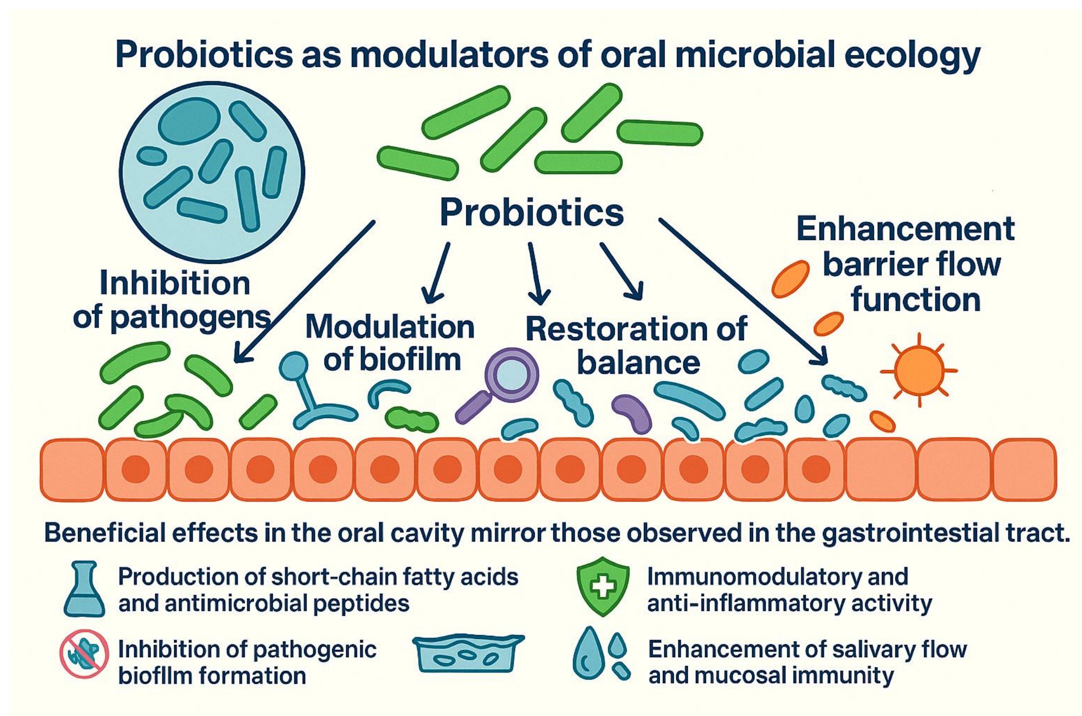


Figure 4. Probiotics as modulators of oral microbial ecology. Probiotics exert multiple beneficial effects within the oral cavity, mirroring mechanisms observed in the gastrointestinal tract, including those summarized in the scheme.

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 [Yu et al., 2024]. Additionally, certain probiotics have been shown to increase salivary levels of secretory immunoglobulin A (sIgA) [Vaisberg et al., 2019; Pahumunto et al., 2019; Ebrahimpour-Koujan et al., 2020], a crucial component of mucosal immunity. sIgA plays 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 [Marcotte and Lavoie, 1998; Ranadheer et al., 2011; Herich, 2017].

1.5. Lactic Acid Bacteria (LAB): Biological Properties and Safety Profile

Lactic acid bacteria (LAB) constitute a heterogeneous group of Gram-positive, catalase-negative, non-spore-forming microorganisms that inhabit a wide range of ecological niches, including plants, animals, and the human body [Duar et al., 2017; De Filippis et al., 2020]. These bacteria are typically anaerobic or facultatively aerobic, exhibit acid tolerance, and primarily rely on fermentative metabolism. Their ability to produce lactic acid and other organic acids has made them indispensable in food preservation and fermentation processes [Yang et al., 2024].

Many LAB strains are classified as "Generally Recognized as Safe" (GRAS) and are widely used as probiotics [Martin and Langella, 2019]. During fermentation, LAB help inhibit the growth of spoilage and pathogenic microorganisms by lowering the environmental pH, thereby enhancing food safety and shelf life [Özogul and Hamed, 2018; Sadiq et al., 2019; Shao et al., 2022].

In recent years, increasing attention has been devoted to the safety evaluation of probiotic LAB for industrial, nutritional, and therapeutic applications [Žuntar et al., 2020; Roe et al., 2022]. Safety assessments vary across regulatory frameworks but generally consider factors such as dosage, duration of toxicity studies, and species-specific responses in animal models [Swanson et al., 2020; Roe et al., 2022].

Moreover, standardized production protocols are essential to ensure the microbiological and biochemical integrity of probiotic formulations.

According to the International Scientific Association for Probiotics and Prebiotics (ISAPP), the development of reliable safety evaluation systems tailored to the unique properties of probiotics is a priority for their responsible use in clinical and industrial settings [Hill et al., 2014]. LAB strains, such as Lactobacillus, are frequently isolated from fermented foods and are also naturally present in the oral and gastrointestinal microbiomes, where they maintain a symbiotic relationship with the host [Khaneghah et al., 2020; Chokset et al., 2023].

Their beneficial effects include [Minj et al, 2021; Bisht et al, 2024]:

- Promotion of commensal bacterial growth
- Inhibition of pathogen adhesion via competition and coaggregation
- Secretion of antimicrobial compounds
- Modulation of immune responses
- Maintenance of microbial homeostasis

Figure 5 summarizes the biological properties, safety profile, and beneficial effects of LAB.

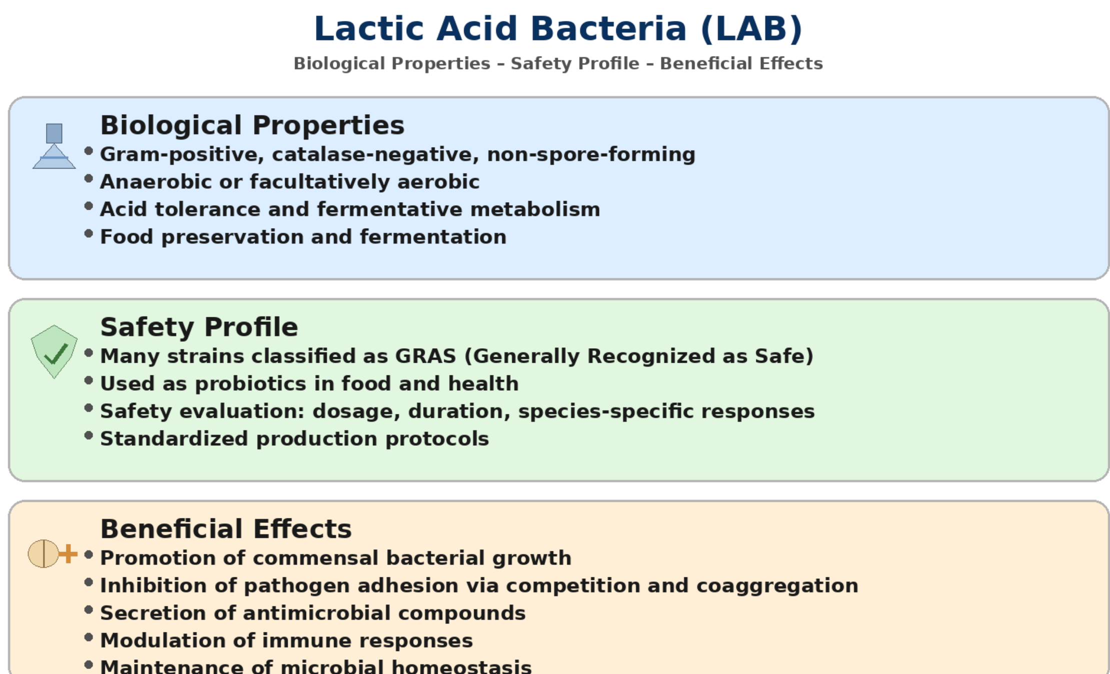


Figure 5. Lactic Acid Bacteria (LAB): Biological properties and safety profile.

1.6. Nitrate-Reducing Bacteria and the Oral–Gut–Systemic Axis

The presence of nitrate-reducing bacteria in the human digestive tract, including the oral cavity, esophagus, and intestines, has garnered significant interest due to their potential impact on systemic health. These bacteria metabolize dietary nitrates, which are secreted into the oral cavity via saliva, converting them into nitrites. Upon swallowing, nitrites are further reduced to nitric oxide (NO) and other nitrogen oxides in the acidic gastric environment. A portion of nitrites is also absorbed into the bloodstream, where they contribute to NO production via enzymatic reduction by hemoglobin and other reductases.

Numerous studies have demonstrated that the increased bioavailability of dietary nitrates and nitrites is associated with cardiovascular benefits, including reduced blood pressure and improved endothelial function. Importantly, antioxidants and polyphenols found in fruits and vegetables mitigate the formation of carcinogenic N-nitroso compounds while enhancing NO synthesis.

In the oral cavity, microbial nitrate reduction contributes to local health by:

- Inhibiting the growth of anaerobic periodontopathogens
- Enhancing resistance to acidification
- Preventing caries development through proton consumption during denitrification
- Utilizing lactic acid and hydrogen sulfide as electron donors, thereby reducing halitosis

The oral microbiome plays a central role in modulating nitrate metabolism, immune responses, and gene expression. A decline in nitrate-reducing bacteria, coupled with an increase in pathogenic species, may explain the observed associations between poor oral health and systemic diseases. Therefore, targeted administration of oral nitrate-reducing probiotics, combined with nitrate-rich dietary interventions, represents a promising strategy to enhance NO bioavailability and maintain systemic homeostasis.

Some LAB strains are particularly noteworthy for their ability to produce nitrate (NO_3^-) and nitrite (NO_2^-) reductase enzymes, which catalyze the conversion of

dietary nitrates into nitrites and subsequently into NO [Ammor and Mayo, 2007; Laranjo et al., 2019]. These enzymatic pathways, along with acid degradation mechanisms and antioxidant interactions (e.g., polyphenols), contribute to the detoxification of nitrosamines and reduction of biogenic amines, thereby lowering the risk of inflammation and carcinogenesis. These enzymes facilitate the conversion of nitrates to nitrites, which subsequently decompose to NO [Xia et al., 2022; Liu et al., 2023; Yuan et al., 2024]. The metabolism and degradation of nitrates and nitrites by LAB involve the action of reductase enzymes, along with acid-degradation mechanisms. Additionally, LAB can utilize extracellular polysaccharides, peroxide-catalyzed oxidation, and antioxidant compounds, such as polyphenols, to enhance their effectiveness in the enzymatic reduction of nitrites [Yuan et al., 2024]. These processes can significantly lower the concentrations of biogenic amines in food products, inhibit the formation of N-nitrosamines, and further degrade N-nitrosamines, thereby reducing their impact on human health and lowering the incidence of diseases, including cancer [Shao et al., 2022; Tan et al., 2023]. Evidence shows that nitrate-reducing bacteria are present in the human digestive system, including the mouth, esophagus, and gastrointestinal tract [Liu et al., 2023]. Numerous studies have highlighted the potential effects of these bacteria on human health and disease, particularly through various disease models [Liu et al., 2023]. This evidence suggests a promising approach for disease prevention and treatment using specific probiotics. In this context, there is growing interest in using specific probiotics as an alternative or adjunct to promote both oral and systemic health. This is especially relevant because the oral cavity serves as the entry point to the respiratory system and the gastrointestinal tract, and it is directly connected to the bloodstream through highly vascularized oral tissues [Pietropaoli et al., 2018; Paul et al., 2021; Hajishengallis and Chavakis, 2021; Barranca-Enríquez and Romo-González, 2022; Botelho et al., 2022; Altamura et al., 2023; Altamura et al., 2024].

Specific LAB isolated from the oral cavity are particularly beneficial due to their ability to produce nitrite from dietary nitrate, which is thought to support systemic

health [Lundberg et al., 2008; Ashworth and Bescos, 2017; Siervo et al., 2018; Rosier et al., 2022; Morou-Bermúdez et al., 2022].

Dietary nitrates are excreted into the oral cavity with saliva, where facultative anaerobic bacteria present in the mouth can convert them into nitrites. Once swallowed, nitrites are spontaneously converted into NO and other nitrogen oxides in the acidic environment of the stomach [Montenegro et al., 2017; Lundberg and Weitzberg, 2022]. However, a small portion is also absorbed directly into the bloodstream, where it is transported to various tissues and reduced by hemoglobin and enzymes [DeMartino et al., 2019]. A plethora of studies indicates that increasing the bioavailability of nitrates and nitrites from exogenous sources is associated with cardiovascular benefits, such as lowering blood pressure [Zhang et al., 2023; Apte et al., 2024]. Notably, the antioxidants and polyphenols found in fruits and vegetables help prevent the formation of carcinogenic N-nitroso compounds from nitrite while simultaneously stimulating NO production [Bak et al., 2025]. In addition to systemic benefits, microbial reduction of nitrates in the mouth promotes oral health [Rosier et al., 2022]. Oral bacteria, by reducing nitrate to nitrite and NO, inhibit the growth of sensitive species, such as anaerobes involved in periodontal diseases [Allaker et al., 2001; Backlund et al., 2014]. Nitrite production has also been shown to increase resilience against salivary acidification in both *in vivo* and *in vitro* studies, thereby preventing caries development [Rosier et al., 2022]. One potential mechanism for this is the consumption of protons during denitrification, or bacterial reduction of nitrite to ammonium.

Additionally, lactic acid, which contributes to oral acidification, and hydrogen sulfide, a volatile compound associated with halitosis, can serve as electron donors in these processes [Rosier et al., 2022]. Moreover, the bioavailability of nitrates and nitrites is significantly influenced by the human oral microbiome, which can affect the host by producing metabolites and modulating immune responses and gene expression [Rajasekaran et al., 2024]. Consequently, a decrease in nitrate-reducing bacteria in the mouth, along with an increase in pathogenic bacteria, may explain the correlation between poor oral health and systemic diseases [Altamura et al., 2023;

Altamura et al., 2024]. Therefore, targeted administration of oral nitrate-reducing bacteria, combined with the consumption of nitrate-rich foods and vegetables, could serve as a practical and effective strategy to enhance NO production and maintain NO homeostasis. This, in turn, may help alleviate disease symptoms and reduce the incidence and severity of certain conditions, offering a novel approach to disease prevention and treatment [Chai et al., 2024]. **Figure 6** summarizes the role of nitrate-reducing bacteria along the oral–gut–systemic axis.

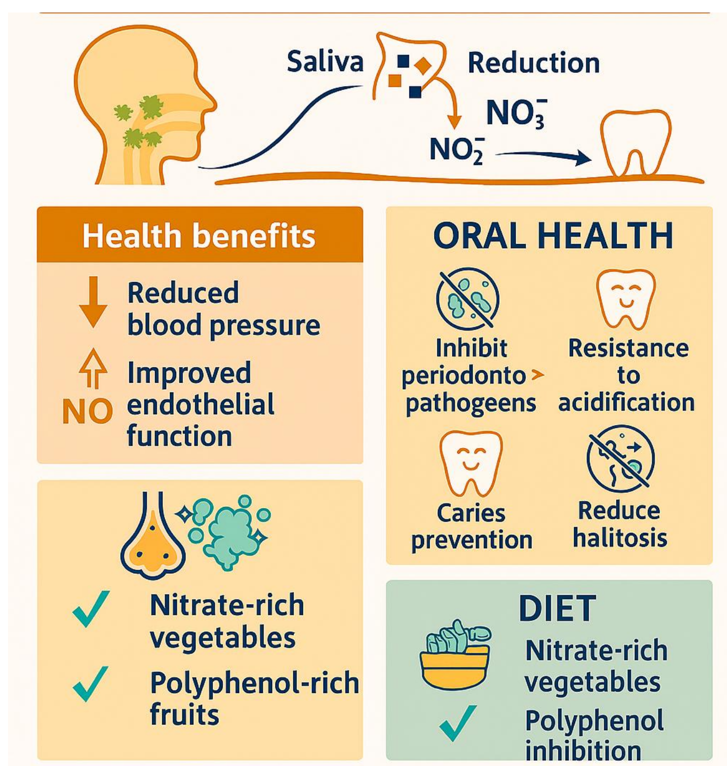


Figure 6. Nitrate-reducing bacteria and the oral-gut-systemic axis. Dietary nitrates secreted into saliva are reduced to nitrites by oral bacteria and further converted to nitric oxide (NO) in the stomach and bloodstream. This pathway contributes to cardiovascular benefits and oral health, including inhibition of anaerobic pathogens, resistance to acidification, caries prevention, and halitosis reduction. Targeted administration of oral nitrate-reducing probiotics, combined with a nitrate- and polyphenol-rich diet, represents a promising strategy for maintaining systemic and oral homeostasis.

1.7. Salivary α-Amylase (sAA): A Functional and Pathological Marker

Human salivary α-amylase (sAA) is an enzyme produced by the salivary glands that plays a crucial role in the initial digestion of starches in the mouth,

catalyzing the conversion of the carbohydrates into shorter oligomers and facilitating their absorption in the digestive tract, where human pancreatic α-amylase (HPA) and α-glucosidases complete the processing of carbohydrates into glucose [Bello-Perez et al., 2020]. While primarily associated with digestive functions, sAA has emerged as a biomarker for oral and systemic conditions [Hashim et al., 2024]. It influences bacterial adhesion and biofilm formation, particularly by facilitating the colonization of amylase-binding streptococci, such as *S. gordonii*, *S. mitis*, *S. parasanguinis*, *S. cristatus*, and *S. salivarius* [Lahiri et al., 2021; Hashim et al., 2024].

These bacteria form stable biofilms capable of metabolizing fermentable sugars (e.g., sucrose, fructose, glucose), producing lactic acid and contributing to enamel demineralization [Kilian and Nyvad, 1990; Douglas, 1994; Sethy et al, 2015; Zaura and Twetman, 2019]. Notably, the binding of bacteria to sAA does not impair its enzymatic activity, allowing for continuous starch hydrolysis and sustained acid production [Scannapieco et al., 1990; Douglas et al., 1992; Sethi et al., 2015].

Given its role in biofilm dynamics and caries pathogenesis, sAA is considered a relevant target for probiotic modulation (**Figure 7**).

Despite the growing body of research on the effects of probiotics on oral health, mechanistic studies examining these beneficial effects remain scarce. Only a few strains, the most well-known being *Levilactobacillus brevis*, have shown direct α-amylase-inhibitory activity *in vitro*, in particular toward porcine pancreatic amylase [Riccia et al., 2007; Campus et al., 2014; Lai et al., 2021; Campus et al., 2025]; however, human data remain scarce. In particular, there is a lack of research investigating the influence of probiotic bacteria on salivary alpha-amylase (sAA) activity, especially in the context of oral diseases such as dental caries.

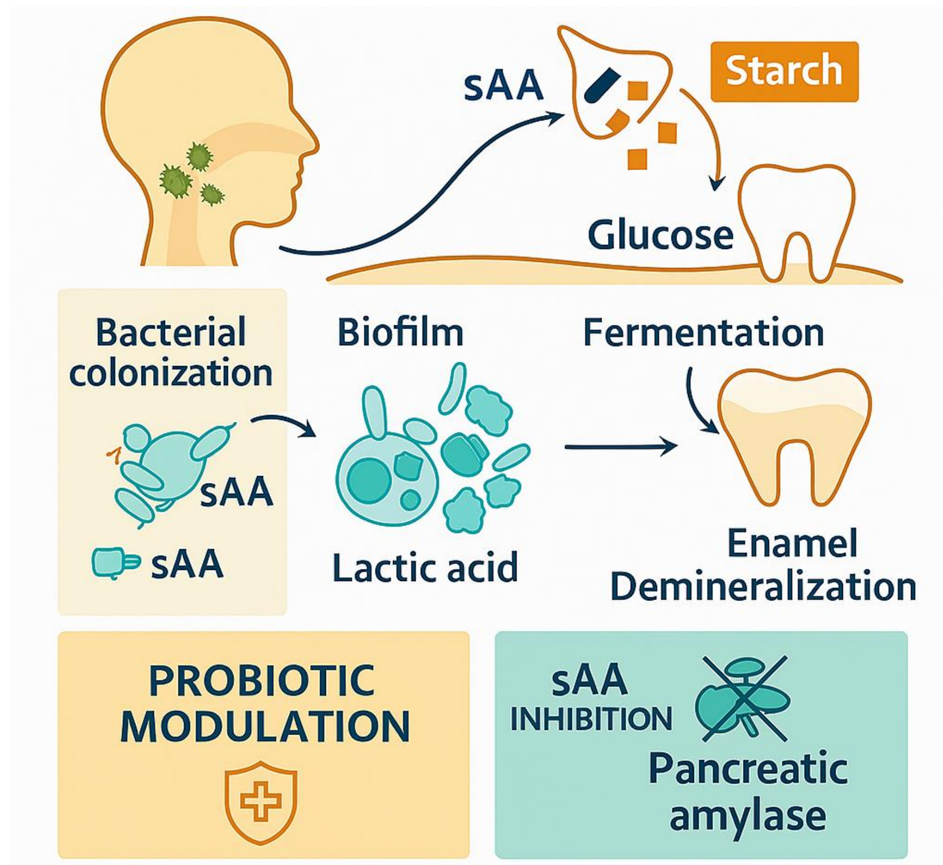


Figure 7. Salivary a-Amylase (sAA): A functional and Pathological Marker. Given its role in biofilm dynamics and caries pathogenesis, sAA is considered a relevant target for probiotic modulation

1.8. Focus on *Levilactobacillus brevis* CD2: A Multifunctional Oral Probiotic

Among the probiotic strains most frequently studied in oral medicine are those belonging to the genera Lactobacillus and Bifidobacterium.

Levilactobacillus brevis, formerly *Lactobacillus brevis* (*L. brevis*) [Todorov et al., 2023], is widely used as a starter culture in the production of various fermented foods [Xia et al., 2017; Cakir et al., 2021; Zhang et al., 2023; Woo et al., 2023]. Moreover, its health-promoting features are well-characterized and exploited as probiotic [Fang et al., 2018]. *L. brevis* CD2 (DSM-27961/CNCM I-5566) is an obligate heterofermentative LAB known for its oral probiotic properties that support various

clinical applications. These properties include the ability to antagonize oral pathogens, inhibit or disrupt pathogen biofilms, colonize oral environments, and provide anti-inflammatory effects. Growing evidence from both *in vitro* and *in vivo* studies supports the use of *L. brevis* CD2 as an effective therapeutic adjuvant or alternative in oral medicine [Tasli et al., 2006; Riccia et al., 2007; Sharma et al., 2012; Niscola et al., 2012; Maekawa and Hajishengallis, 2014; Campus et al., 2014; Lee et al., 2015; Shah et al., 2017; Lai et al., 2021; Altamura et al., 2024; Altamura et al., 2025].

Notably, *L. brevis* CD2 (CNCM I-5566) has demonstrated multifunctional properties in various clinical contexts, including dental caries, gingivitis, periodontitis, oral mucositis, Behçet's disease, and aphthous ulcers.

Beyond its capacity to compete with oral pathogens, *L. brevis* CD2 has been shown to modulate host inflammatory responses by inhibiting the nitric oxide synthase 2 (NOS2) pathway, thereby reducing NO synthesis, a potent mediator of inflammation. The strain's beneficial effects are largely attributed to its high arginine deiminase (ADI) expression, an enzyme that catalyzes the conversion of arginine into citrulline and ammonia (NH_3). In aqueous environments, NH_3 forms ammonium ions (NH_4^+), which help maintain physiological salivary pH and prevent acidification and enamel demineralization. Furthermore, ADI competes with arginase and NOS2 for arginine, thereby inhibiting the synthesis of polyamines (e.g., putrescine and spermidine), which are involved in halitosis and tumor proliferation, as well as NO associated with periodontal inflammation. Recent experimental data from our group, using a pH cycling model to simulate mineral loss, have demonstrated that *L. brevis* CD2 effectively protects enamel surfaces from demineralizing agents and enhances resistance to acid-induced damage. Despite the growing body of evidence supporting the clinical utility of *L. brevis* CD2, further robust studies were needed to confirm its preventive efficacy in oral health.

2. Objectives and Aims of the Study

This thesis aimed to evaluate the therapeutic and preventive potential of the probiotic strain *L. brevis* CD2 (CNCM I-5566) in promoting oral health through an integrated approach encompassing *in vivo, ex vivo,* and *in vitro* investigations.

The specific objectives were:

1. Clinical Evaluation

   To assess the effects of a four-week supplementation with *L. brevis* CD2-containing lozenges on key oral health parameters in healthy adults, including:

   - Bleeding on probing (BoP)
   - Plaque index (PI)

2. Salivary Analysis

   To investigate changes in salivary composition and function, including:

   - *Salivary parameters:*
     - Salivation rate
     - pH
     - Buffering capacity
   - *Biochemical markers:*
     - Glucose
     - D-lactate
     - Secretory IgA (sIgA)
     - Nitrate and nitrite levels (as indicators of oral nitrate-reducing capacity)
     - α-amylase activity (salivary and pancreatic)

3. *Ex Vivo* Evaluation

   To determine the nitrate-reducing capacity of *L. brevis* CD2 using salivary samples:

   - Under baseline conditions
   - After the addition of exogenous nitrates

4. Conceptual Contribution:

   To contribute to the understanding of probiotic applications in oral health, proposing *L. brevis* CD2 as a promising candidate for microbiome-based preventive and therapeutic strategies.

3. Research Design, Data Collection, and Analytical Methods

3.1. Study Design and Ethical Approval

This double-blind, randomized controlled trial (RCT) was conducted between November 2022 and November 2023 to evaluate the effectiveness of a four-week intervention with the probiotic *Levilactobacillus brevis* CD2 (CNCM I-5566) in reducing the full-mouth bleeding on probing (BoP) score in a population of otherwise healthy adults presenting with at least localized gingivitis (BoP ≥ 10%). The full-mouth BoP score was designated as the primary outcome, while the full-mouth plaque index (PI) and various salivary parameters and biomarkers, including salivation rate, pH, buffering capacity, salivary glucose, D-lactate, secretory immunoglobulin A (sIgA), nitrate reduction activity, and α-amylase levels were considered co-primary outcomes. The study received ethical approval from the Internal Review Board of the University of L'Aquila (Italy) under Protocol No. 48/2022, dated November 22, 2022, and was registered on ClinicalTrials.gov (NCT06457724). The research was conducted without external funding and adhered to the ethical principles outlined in the Declaration of Helsinki for human clinical studies [World Medical Association. World Medical Association Declaration of Helsinki, 2013], as well as the CONSORT 2010 guidelines for reporting randomized clinical trials [Schulz et al., 2010]. A detailed overview of visit procedures and study timelines is provided in **Table 1**.

Table 1. Study schedule

Elegibility Assessment	Screening Visit	Baseline Visit (T0)	Four Weeks (T1)	Six Weeks (T2)
Inclusion	X			
Exclusion	X			
Oral hygiene instructions	X			
Instimulated saliva collection		X	X	X
Salivation rate measurement		X	X	X
DMFT	X			
Salivary pH		X	X	X
Salivary buffering capacity		X	X	X

PI	X	X	X	X
BoP	X	X	X	X
sIgA		X	X	X
Salivary glucose		X	X	X
Salivary D-lactate		X	X	X
Nitrate-reduction activity		X	X	X
sAA activity		X	X	X

Legend: DMFT-decayed, missing, and filled teeth; PI-plaque index; BoP-bleeding on probing; sIgA-secretory immunoglobulin A; sAA-salivary α-amylase

3.2. Questionnaire and Sampling Criteria

Subjects attending the Dental Clinic at the University of L'Aquila were invited to participate in the study. They were informed about the study's objectives, procedures, and the opportunity to take part. Information regarding participants' age, general health status, smoking habits, and oral hygiene practices was collected via a questionnaire and securely stored in a password-protected electronic archive. A total of 30 healthy adult volunteers, aged 20 to 75 years, were enrolled and assigned to the intervention groups by one of the study authors. During the screening visit, a comprehensive full-mouth clinical examination was conducted to assess the number of DMFT, PI, and BoP scores. BoP was recorded as a binary outcome (yes/no) at 6 sites per tooth across 28 teeth, yielding 168 measurements per subject. Written informed consent was obtained from all participants for both inclusion in the study and publication of data for research and educational purposes. No compensation was provided, and participants did not receive any specific recommendations regarding personal oral hygiene practices during the trial period. Eligibility criteria required participants to be between 20 and 75 years of age, have a permanent dentition with more than 20 teeth, and present a full-mouth BoP score of 10% or higher. Individuals were excluded if they declined to sign the informed consent, had used supplements or lozenges containing probiotics or prebiotics within three weeks before the study, had taken antibiotics within one month before the study, were undergoing orthodontic or prosthetic treatment, or had known allergies to any components of the study treatments. Additional exclusion criteria included the use of other hygiene products, immunostimulants, antibacterials, probiotics, or prebiotics during the study

period, refusal to take the assigned lozenges, or failure to attend scheduled follow-up visits.

3.3. Sample Size Calculation

The sample size for this RCT was determined based on data from a previous exploratory case-control study (unpublished). The primary outcome measure was the full-mouth BoP score. To detect a reduction in BoP from 25.2% in the placebo group to 11.6% in the *L. brevis* CD2 group (SD=12.1), with 80% statistical power and a significance level of 5%, a total of 26 participants (13 per group) was required. This calculation ensured that the study was adequately powered to detect a statistically significant difference between the treatment and placebo groups, should one exist. The sample size was computed using standard formulas for comparing continuous outcomes in clinical trials, accounting for the expected effect size and variability [Julious, 2004; Pocock, 2013].

3.4. Randomization Procedures and Interventions

Eligible participants were randomized in a 1:1 ratio using a block randomization method (sealedenvelope.com), with block sizes of 4, 6, and 8, and a list length of 36. Allocation concealment was ensured by using sealed, opaque envelopes containing the group assignment (intervention or placebo). Once a participant provided informed consent, an envelope was opened to reveal the assigned treatment. To maintain blinding for both participants and clinical investigators, an external researcher, who was not involved in any clinical or statistical procedures, was responsible for opening the envelopes and distributing the treatments. Of the 36 individuals screened, 30 met the eligibility criteria and were enrolled in the study (15 females and 15 males) (**Figure 8**). These participants were randomly assigned to one of two groups: the *L. brevis* CD2 group (n = 15; 7 females, 8 males), which received lozenges containing the probiotic, and the placebo group (n = 15; 8 females, 7 males), which received lozenges identical in taste, colour, texture, and size but without active bacteria. Each participant received a container of lozenges at enrolment. Both participants and researchers were blinded to the type of lozenges

administered. All 30 participants completed the study protocol, and no adverse effects were reported.

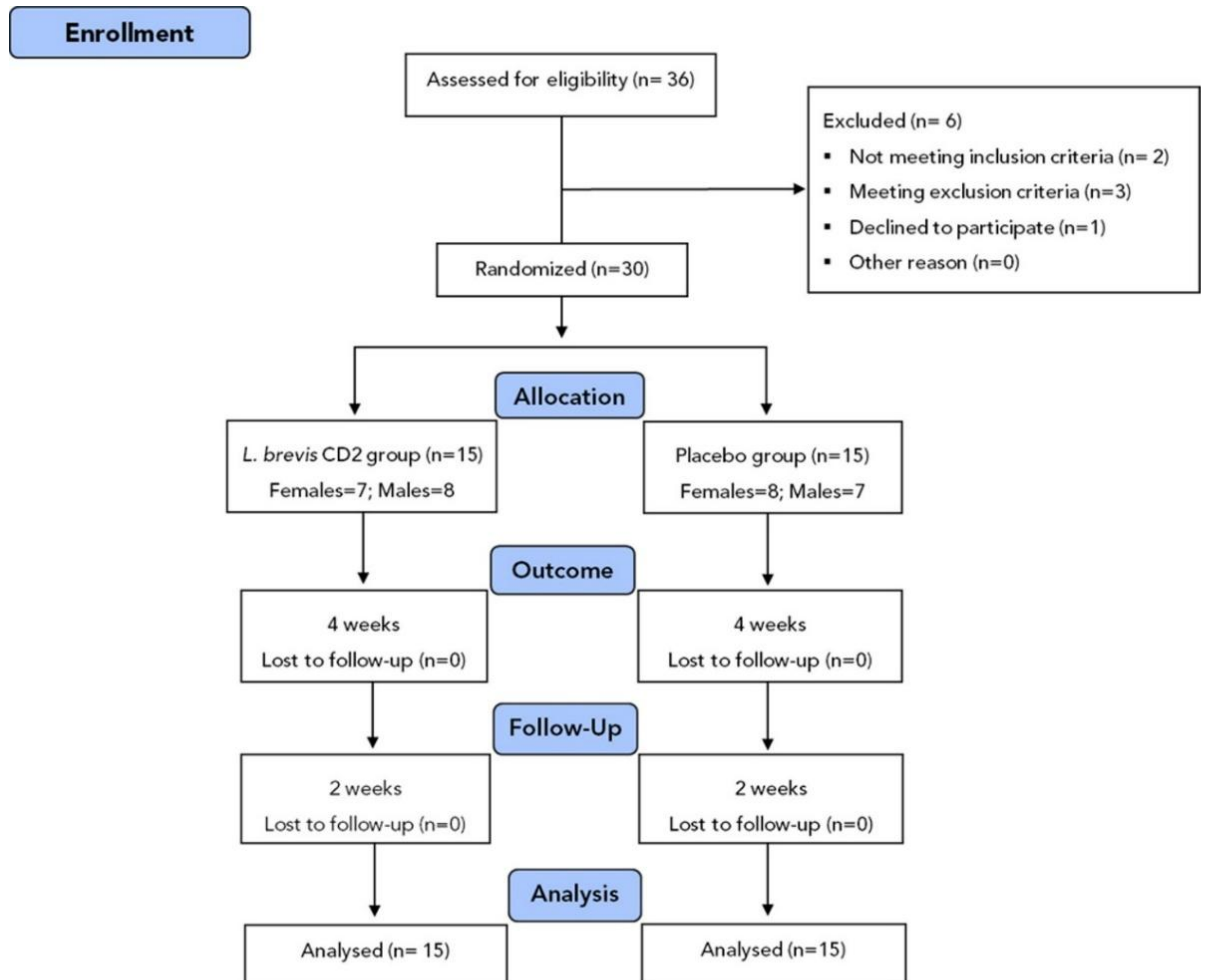


Figure 8. Consort flowchart.

3.5. Intervention

All participants were instructed to take four lozenges per day for four weeks, receiving the active intervention (*L. brevis* CD2, CNCM I-5566 $\geq 1 \times 10^9$ CFU/lozenge; Mucomixx Lot. N° 2301401; EOS2021 Srl, Rome, Italy) or the placebo. The detailed composition of both the probiotic and placebo lozenges is provided in Table 2. Following the four-week intervention period, participants underwent a two-week washout phase during which they abstained from taking

lozenges. This washout period was implemented to evaluate the stability and persistence of the effects observed during the intervention.

Table 2. Dietary supplement composition.

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

3.6. Saliva Sample Collection and Flow Rate Measurements

To collect unstimulated salivary samples, participants were instructed to refrain from eating, drinking, smoking, or performing any oral hygiene procedures for at least 90 minutes before sampling. All measurements were conducted between 9:00 and 12:00 a.m. to minimize diurnal variations in salivary output. Participants were comfortably seated in a dental chair and, after five minutes of relaxation, were trained to refrain from swallowing saliva. They were then asked to lean forward and expectorate all saliva produced over 5 minutes into a pre-labelled, pre-weighed 50 mL sterile polypropylene tube. The total volume of saliva collected was recorded.

Salivary flow rate was calculated using the following formula: Salivation rate (mL/min) = saliva volume (mL)/collection time (min).

Each saliva sample was subsequently thawed and centrifuged at 500 × g for 10 minutes. The resulting supernatants were aliquoted into 1.5 mL tubes and stored at −80 °C until further processing.

3.7. Clinical Evaluations

Clinical examinations were conducted by one of the study authors. Gingival condition was assessed using the full-mouth BoP score, calculated as the percentage of bleeding sites out of six sites per tooth (excluding third molars). BoP presence or absence was recorded using a millimeter probe (UNC-15 probe, PCPUNC15, Hu-Friedy, North Carolina, USA), which was gently inserted between the tooth and gingiva at four sites per tooth—buccal, mesial, distal, and palatal/lingual—with an

approximate pressure of 20 N [Ainamo and Bay, 1975]. The extent of BoP was determined by dividing the number of bleeding sites by the total number of probed sites and expressing the result as a percentage. Gingival status was classified dichotomously: non-bleeding gums (BoP<10%) and bleeding gums (BoP≥10%) [Silness and Loe, 1964]. PI was defined as the percentage of sites with visible dental plaque, assessed at four sites per tooth, excluding third molars [Caton et al., 2018]. Each surface—buccal, lingual, mesial, and distal—was scored as follows: 0= no plaque; 1= a thin film of plaque adhering to the free gingival margin and adjacent tooth area, 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 from each examined surface were summed and divided by the total number of surfaces assessed.

3.8. Estimation of pH and Buffering Capacity of Saliva

Immediately after collection, 0.5 mL of each saliva sample was transferred into a sterile tube. The pH was measured using a digital pH meter (Mettler Toledo, Columbus, Ohio, USA). The buffering capacity of saliva was assessed using the Ericsson method [Ericsson, 1959]. For this analysis, 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 left to stand for 10 minutes. The final pH was then recorded using the same digital pH meter.

3.9. Quantification of Salivary Biomarkers

3.9.1. Glucose

The quantitative measurement of salivary glucose concentration was performed using a colorimetric glucose assay kit (Cell Biolabs, San Diego, CA, USA), following the manufacturer's instructions. Absorbance was measured spectrophotometrically using a microplate reader (Bio-Rad Laboratories, Hercules, CA, USA) at 550 nm. Glucose concentration was calculated based on a standard curve and expressed in mg/dL.

3.9.2. D-Lactate

Salivary D-lactate levels were measured using a D-lactic acid/lactate colorimetric assay kit (Immunological Sciences, Rome, Italy), following the manufacturer’s instructions. Absorbance was recorded using a spectrophotometric microplate reader (Bio-Rad Laboratories) at a wavelength of 530 nm. D-lactate concentration was calculated based on the standard curve and expressed in mmol/dL.

3.9.3. Secretory Immunoglobulin A (sIgA)

sIgA levels were quantified using an ELISA assay kit (Immunological Sciences), following the manufacturer’s instructions. Absorbance was measured using an ELISA reader at 570 nm. The concentration of sIgA was expressed in µg/mL.

3.9.4. Whole-Mouth Nitrate Reduction

To assess oral nitrate-reducing capacity, participants were instructed to rinse with 10 mL of water containing 80 µM sodium nitrate for 5 minutes, following a previously validated protocol [Ashworth et al, 2019]. The rinse was collected into sterile Falcon tubes, centrifuged at 2150 × g for 10 minutes at 4 °C, and the supernatant was stored at −80 °C until analysis. Absolute nitrite concentrations were measured using the Griess reaction, as described above. This assay was performed at three time points: before treatment (T0), after 4 weeks of intervention (T1), and after a 2-week washout period (T2) in both the probiotic and placebo groups.

3.10. *In Vitro* Experiments

3.10.1. Preparation of L. brevis CD2 lysate

Mucomixx lozenges (Lot No. 2301401; EOS2021 Srl, Rome, Italy), each containing 10^9 CFU of *L. brevis* CD2 (DSM-27961/CNCM I-5566), were dissolved in phosphate-buffered saline (PBS; EuroClone, UK). The suspension was centrifuged at 8600×g, washed twice, resuspended in PBS, and sonicated using a Vibracell

sonicator (Sonic and Materials, USA). Cell disruption was verified by measuring absorbance at 590 nm (Eppendorf, Germany) before and after sonication. Total protein concentration was determined using a DC Protein Assay (Bio-Rad Laboratories, USA).

Additional lysates were prepared by suspending lyophilized *L. brevis* CD2 (provided by Prof. Claudio De Simone) in PBS (1 g/10 mL) and centrifuging at 17,949 × g. The resulting supernatant was filtered through a 0.22 μm membrane (Corning Inc., USA) to remove intact bacteria. Protein content was quantified as above.

3.10.2. Nitrate-Reducing Activity in L. brevis CD2 Lysate

The nitrate-reducing capacity of the probiotic lysate was determined as previously described [Arias-Negrete et al., 2004]. A 100 μL of 16 mM sodium nitrate (Sigma-Aldrich, St. Louis, MO, USA) in PBS was combined with increasing concentrations of *L. brevis* CD2 lysate, ranging from 0 to 6400 μg protein/mL. The total volume was then adjusted to 200 μL with PBS, resulting in a final sodium nitrate concentration of 8 mM. Samples were incubated at 37°C for 3 hours in a thermomixer (Eppendorf, Hamburg, Germany), followed by centrifugation at 14,000 × g.

The nitrite concentration in the supernatant was measured using the Griess reaction, as described below.

In an additional set of experiments, the nitrate-reducing activity of *L. brevis* CD2 lysate at a fixed concentration of 1600 μg protein/mL was evaluated after 3 h of incubation with varying sodium nitrate concentrations (0-8 mM). In the enzyme activity assays, the nitrate reductase provided in the Nitrate/Nitrite Assay Kit (Sigma-Aldrich) served as a positive control. A mixture of 10 μL nitrate reductase and 10 μL co-factor solution was incubated under the experimental conditions described previously for 3 h at 37 °C. For comparison with the experimental probiotic, the enzyme activity was measured in micromoles of nitrite produced per minute (units of nitrate reductase).

3.10.3. Amylase Activity Assay

sAA activity was quantified using a colorimetric assay kit (Amylase Activity Assay Kit, Sigma-Aldrich, St. Louis, MO, USA), following the manufacturer's protocol. The assay is based on the enzymatic cleavage of the synthetic substrate ethylidene-pNP-G7, with one unit of activity defined as the amount of amylase required to release 1.0 µmol of p-nitrophenol per minute at 25 °C.

To investigate the potential inhibitory effect of *L. brevis* CD2 on amylase activity, *in vitro* experiments were conducted using purified human sAA (α-amylase from human saliva, Type XIII-A, ~1000 U/mg protein, Sigma-Aldrich) at a final concentration of 60 mU/mL, and human pancreatic α-amylase (HPA, ~290 U/mg protein, USBiological Life Sciences, MA, USA) at 15 mU/mL. Enzymes were incubated for 10 minutes at room temperature with or without *L. brevis* CD2 lysate at increasing protein concentrations (0.25, 0.5, and 1 mg/mL). Amylase activity was then measured using the same colorimetric assay, allowing evaluation of the dose-dependent inhibitory effects of the probiotic lysate.

3.10.4. Amylase-binding Assay

Amylase-binding protein precipitation was performed using previously described protocols [Li et al., 2002; Chaudhuri et al., 2007; Vorrasi et al., 2010; Nikitkova et al., 2012; Maddi et al., 2014], with minor modifications. Briefly, *L. brevis* CD2 lysate (1 mg/mL) was incubated with either sAA or HPA at 100 µg/mL in PBS for 2 hours at 25 °C under agitation. Control samples containing sAA, HPA, or *L. brevis* CD2 alone were processed under identical conditions.

Following incubation, precipitates were collected by centrifugation at 5000×g for 15 minutes at 4°C. Supernatants were discarded, and the pellets were washed with PBS. Then, they were solubilized in loading buffer and boiled for 5 minutes to denature the proteins. Samples were subsequently loaded onto 10% SDS-PAGE gels for electrophoresis. Positive controls included *L. brevis* CD2 (25 µg), sAA, and HPA (1 or 2 µg, as indicated in figure legends), which were not subjected to incubation.

Protein bands were visualized using 0.1% (w/v) Coomassie Brilliant Blue R-250 staining in 40% (v/v) methanol and 1% (v/v) acetic acid. Imaging was performed using the Alliance system (UVITEC, Cambridge, UK). Molecular weight analysis

was conducted using Alliance software by comparing sample bands to a standard curve generated from the Broad Multi-Color Pre-Stained Protein Standard (GenScript, New Jersey, USA), which was loaded on the same gel.

To confirm the identity of α-amylase, proteins resolved by SDS-PAGE were transferred to nitrocellulose membranes, blocked with 5% non-fat dry milk, and incubated with either a mouse monoclonal antibody against human sAA or HPA (Novus Biologicals, Colorado, USA), both at a 1:1000 dilution. Detection was performed using an HRP-conjugated goat anti-mouse IgG secondary antibody (Immunological Sciences, Rome, Italy), and immunoreactive bands were visualized using the Alliance chemiluminescence documentation system.

3.11. *Ex Vivo* Experiments

3.11.1. Nitrate-Reducing Activity in Human Saliva Samples

Saliva samples from six participants (4 females and 2 males, aged 25–43 years) enrolled in the clinical trial were used for *ex vivo* analyses. For detailed information regarding the clinical trial procedures and ethical considerations, refer to Section 3.1.

3.11.2. Salivary Nitrate Reduction Test

The nitrate-reducing capacity in saliva was assessed by incubating samples for 3 h at 37 °C in a thermomixer, either in the presence or absence of 8 mM nitrate, as previously described [Rosier et al., 2024]. For the assay, 25 µL of water containing 80 mM sodium nitrate (Sigma-Aldrich) was added to 225 µL of saliva, with or without probiotic lysate at 1600 µg protein/mL. The concentration of accumulated nitrites was quantified as described below.

3.11.3. Nitrite Level Assay

Nitrite levels were quantified using the Griess reaction with a nitrite assay kit from Sigma-Aldrich. Samples, either probiotic lysates or treated saliva, were loaded into a 96-well microplate following the manufacturer's protocol. The absorbance was measured by spectrophotometric reading at 550 nm using a microplate reader (Bio-

Rad Laboratories). The nitrite content of each sample was evaluated using a standard curve generated by linear regression of a sodium nitrite solution, expressed in µM.

3.11.4. pH and Buffering Capacity Assessment in Human Saliva Samples

To investigate the impact of nitrate-reducing activity and treatment conditions on salivary parameters, pH and buffering capacity were assessed both before and after a 3-hour *in vitro* incubation. Measurements were performed using the same instrumentation and protocols described in section 3.8. Specifically, the Ericsson method was employed to evaluate buffering capacity, and pH values were recorded with a digital pH meter (Mettler Toledo, Columbus, OH, USA). This approach allowed for comparative analysis of salivary acid-base properties in response to experimental treatments.

4. Statistical Analysis

To appropriately analyse the data collected from the different experimental components, distinct statistical approaches were employed for the *in vivo*, *in vitro*, and *ex vivo* analyses, reflecting the specific nature and structure of the datasets.

For the *in vivo* clinical data, the primary outcome was the change in full-mouth BoP from baseline (T0) to four weeks post-treatment (T1). Change scores (ΔBoP) were calculated for each participant, and descriptive statistics, including means and standard deviations, were computed for both treatment and placebo groups. Data visualization was performed using bar plots and scatterplots, with results reported as means and standard errors unless otherwise specified. Given the non-normal distribution of the data, confirmed by the Shapiro-Wilk test on ANCOVA residuals ($W=0.9438$, $p<0.001$), the Wilcoxon rank-sum test was used to compare change scores between groups. To ensure robustness, an inverse hyperbolic sine (ASINH) transformation was applied to the data, allowing negative values to be included and re-assessing normality. Additionally, robust regression analysis was conducted to minimize the influence of outliers, and bootstrapping with 1000 replications was used to estimate confidence intervals for the treatment effect. All

statistical analyses were performed using R software (version 4.3.2) for ARM M1 processors.

For the *in vitro* and *ex vivo* data, statistical analysis was performed using GraphPad Prism version 8.02 (GraphPad Software, San Diego, CA, USA). Data normality was assessed using the Shapiro–Wilk test. Non-normally distributed data were analysed using the two-sided paired Wilcoxon signed-rank test for within-group comparisons, and the two-sided Mann–Whitney test for between-group comparisons. Oral nitrate-reducing capacity in saliva samples from the *L. brevis* and placebo groups was analysed using a two-way repeated measures ANOVA followed by Bonferroni post hoc testing. Data were expressed as means ± SD or SE, or as medians with interquartile ranges (IQR, 25th–75th percentile), as specified in figure legends. A p-value<0.05 was considered statistically significant. Additionally, to visualize the variation in salivary parameters after incubation with or without the *L. brevis* CD2 lysate for 3 hours, a heat map was generated using z-score normalized data.

In a separate set of analyses, the normality of distributions was again assessed using the Shapiro–Wilk test. Parametric data were reported as means ± SD or SE, while non-parametric data were expressed as medians with interquartile ranges. Categorical variables were presented as counts and percentages. Fisher's exact test and the Wilcoxon rank-sum test were used to compare gender and age distributions between groups. Differences in sAA levels within and between the *L. brevis* and placebo groups were evaluated using a two-way ANOVA followed by Bonferroni post hoc testing. The *in vitro* effects of *L. brevis* on sAA and HPA activity were analysed using a one-way ANOVA followed by Dunnett's post hoc test. The significance threshold for all analyses was set at $\alpha=0.05$, and all computations were performed using GraphPad Prism version 8.02.

5. RESULTS

5.1. Baseline Characteristics

Baseline demographic and clinical characteristics of the study population are presented in **Table 3**, stratified by treatment allocation (*L. brevis* CD2 vs. placebo).

A total of 30 participants were enrolled, evenly distributed across the two study arms (n=15 per group). The gender distribution was balanced, with 46.7% females in the *L. brevis* CD2 group and 53.3% in the placebo group. Age distribution was predominantly between 24 and 43 years (n=24), while six participants, three from each group, were aged 51-70 years. The mean age was comparable across groups: 37.9 years (SD=14.2) in the probiotic group and 38.1 years (SD=13.2) in the placebo group. Smoking status was equally represented, with 46.7% non-smokers and 53.3% smokers in both groups. No statistically significant differences were observed between the groups with respect to baseline variables, including BoP and PI scores, salivary flow rate, pH, buffering capacity, glucose concentration, D-lactate levels, sIgA concentrations (all $p>0.05$).

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
Non smokers (%)	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
Oral nitrate-reducing capacity (µM) (mean (SD))	18.42 (6.13)	17.85 (5.98)	0.734
sAA (U/mL) (mean (SD))	112.5 (25.4)	118.3 (27.1)	0.612

5.2. Effects of L. brevis CD2-Containing Lozenges on Full-Mouth BoP and PI Scores

The administration of *L. brevis* CD2 lozenges over four weeks resulted in a statistically significant reduction in full-mouth BoP scores compared to baseline values (*p<0.0001*) (**Figure 9A**). This improvement was sustained even after a two-week washout period (*p<0.0001*), indicating a prolonged therapeutic effect of the probiotic on gingival inflammation. In contrast, the placebo group did not exhibit any significant changes in BoP scores at either post-treatment or follow-up assessments (**Figure 9A**). Intergroup comparisons revealed that BoP scores were significantly lower in the *L. brevis* CD2 group than in the placebo group at both T1 (post-treatment) and T2 (washout) time points (*p<0.001*), confirming the efficacy of the probiotic intervention (**Figure 9B**).

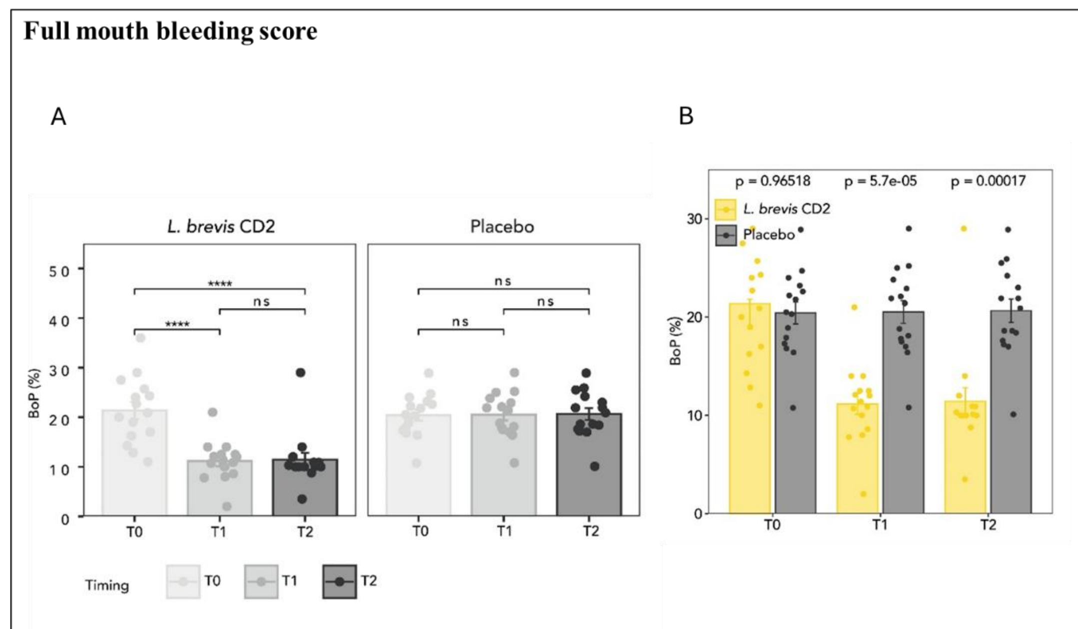


Figure 9. Evaluation of full mouth bleeding score in *L. brevis* CD2 and placebo groups at T0, T1, and T2. BoP score expressed as mean ± SE. ns = non-significant, ****p<0.001*, and *****p<0.0001*.

A similar trend was observed for PI scores. Following the four-week probiotic regimen, PI values were significantly reduced compared with baseline (*p<0.0001*). They remained stable during the washout period (*p<0.0001*), suggesting a durable

effect on plaque accumulation (**Figure 10A**). No significant changes in PI scores were detected in the placebo group at any time point. Between-group comparisons further demonstrated that PI scores were markedly lower in the probiotic group than in the placebo group at both T1 and T2 ($p<0.0001$) (**Figure 10B**).

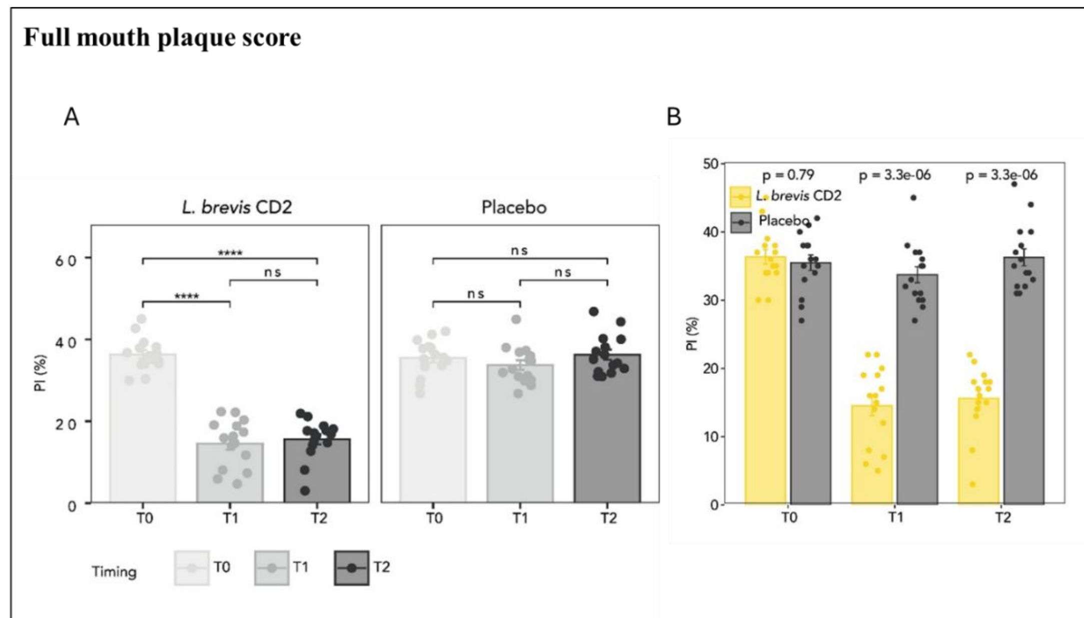


Figure 10. Evaluation of full mouth plaque score in *L. brevis* CD2 and placebo groups at T0, T1, and T2. PI scores expressed as mean ± SE. ns = non-significant, and ****$p<0.0001$.

Further analysis using quadrant plots (**Figure 11A** and **11B**) revealed a significant positive correlation between changes in BoP and PI scores at both T1 (R=0.72) and T2 (R=0.67). Most data points in the *L. brevis* CD2 group were in the green quadrant, indicating concurrent improvements in both parameters.

To explore the potential mediating role of PI in the observed changes in BoP, a mediation analysis was conducted. The model assessed the relationship between treatment assignment (x), change in PI (ΔPI) as the mediator (m), and change in BoP (ΔBoP) as the outcome (y) (Figure 8C). The total effect of the treatment on ΔBoP was 10.3 ($p<0.001$), while the direct effect, controlling for PI, was 10.4 ($p<0.001$). The indirect effect (ab) through PI was −0.12, with a confidence interval that did not include zero, indicating no statistical significance. These findings suggest that the effect of *L. brevis* CD2 on BoP reduction was primarily direct, rather than mediated by changes in plaque accumulation.

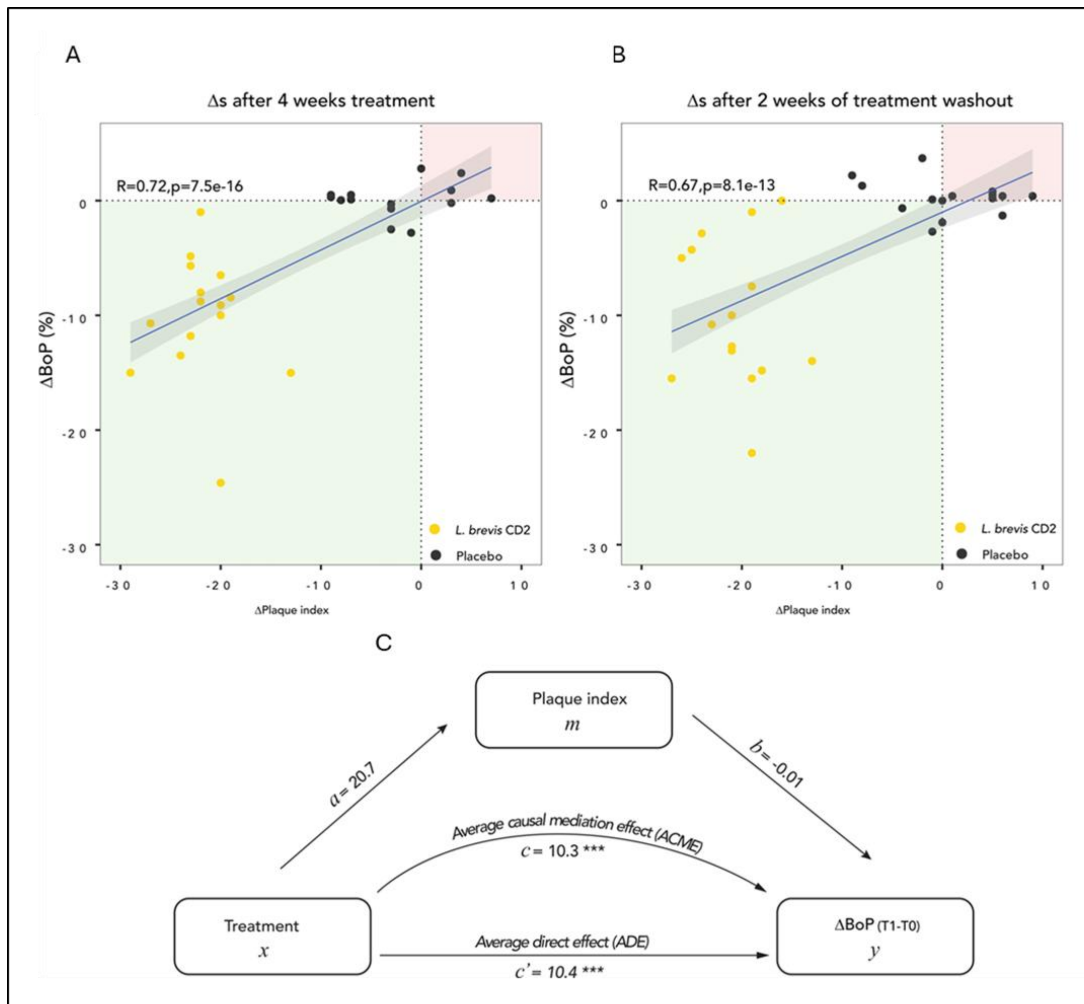


Figure 11. Quadrant Analyses depicting changes (Δ) in BoP and PI scores at T1 (A) and T2 (B). Each dot represents an individual. Dots in the green and red areas represent, respectively, improvements or worsenings in the scores. Correlation coefficient (R) and *p*-value of the relationship between the changes in BoP and PI scores. Mediation Analysis is also shown (C). ns = non-significant, ****p<0.001, and ****p<0.0001.*

5.3. 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*) (**Figure 12A**). 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*) (**Figure 12B**).

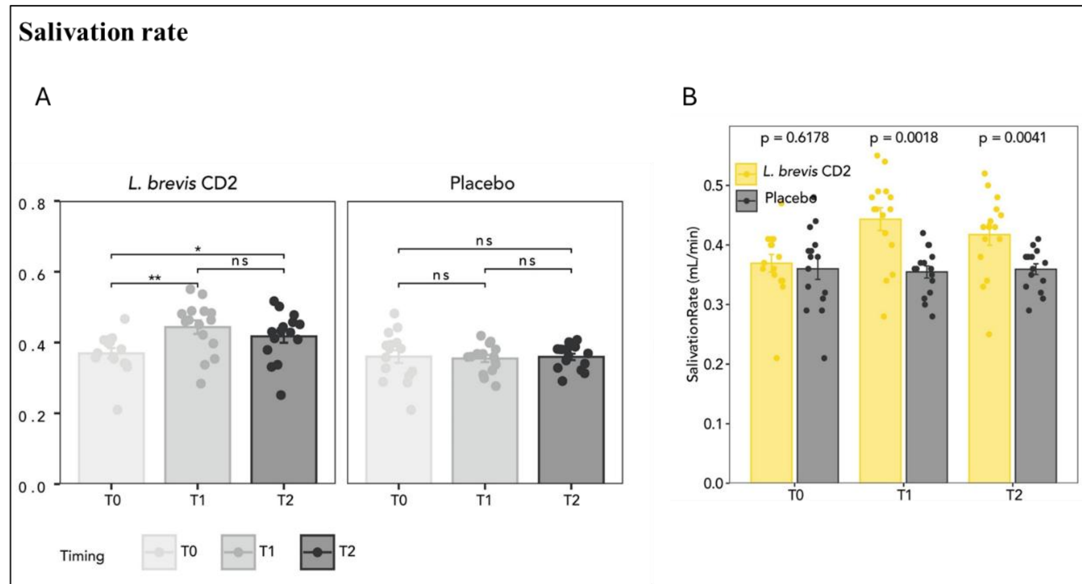


Figure 12. Evaluation of salivation rate in *L. brevis* CD2 and placebo groups at T0, T1, and T2. Salivation rate expressed as mean ± SE. ns = non-significant, **p<0.05*, and ***p<0.01*.

The four-week treatment with *L. brevis* CD2 did not significantly alter salivary pH compared to baseline, and the pH did not change even after the two-week washout (**Figure 13A**). In subjects treated with placebo, no significant changes in salivary pH were detected at T1 or T2 compared with baseline.

Regarding differences between groups, the probiotic group showed a higher pH at T1 than the placebo group, although this difference was not significant (**Figure 13B**). Although the analysis did not show differences in salivary pH between T0 and T1, a statistical difference was observed at T2 (*p<0.05*).

Salivary pH

Figure 13. Evaluation of salivary pH in *L. brevis* CD2 and placebo groups at T0, T1, and T2. Salivary pH expressed as mean ± SE. ns = non-significant, *$p<0.05$.

After four weeks of *L. brevis* CD2 treatment, the buffering capacity significantly increased compared to baseline ($p<0.0001$) (**Figure 14A**), and the values remained substantially higher also at washout ($p<0.0001$), demonstrating the prolonged effect of the probiotic. No significant changes were observed in the placebo group at any time point. Between-group analysis revealed that buffering capacity was significantly higher in the probiotic group than in the placebo group at both T1 and T2 ($p<0.0001$) (**Figure 14B**).

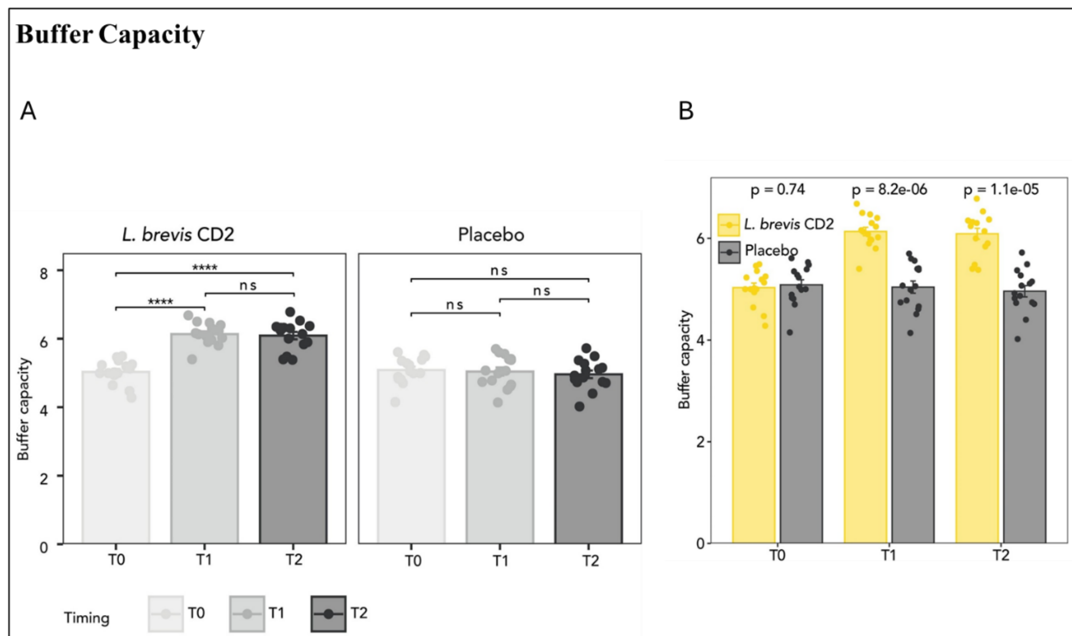


Figure 14. Evaluation of buffer capacity in *L. brevis* CD2 and placebo groups at T0, T1, and T2. Buffer capacity expressed as mean ± SE. ns = non-significant, and ****$p<0.0001$.

5.4. Effects of L. brevis CD2-Containing Lozenges on Salivary Biomarkers

After four weeks of *L. brevis* CD2 supplementation, a reduction in salivary glucose levels was observed compared to baseline. Although this decrease did not reach statistical significance immediately after treatment, it became significant following the two-week washout period ($p<0.05$) (**Figure 15A**). In contrast, no significant changes in salivary glucose concentrations were detected in the placebo group at any time point. When comparing the two groups, participants receiving *L. brevis* CD2 exhibited significantly lower salivary glucose levels than those in the placebo group at the end of treatment ($p<0.05$) and, more markedly, after the washout period ($p<0.01$) (**Figure 15B**).

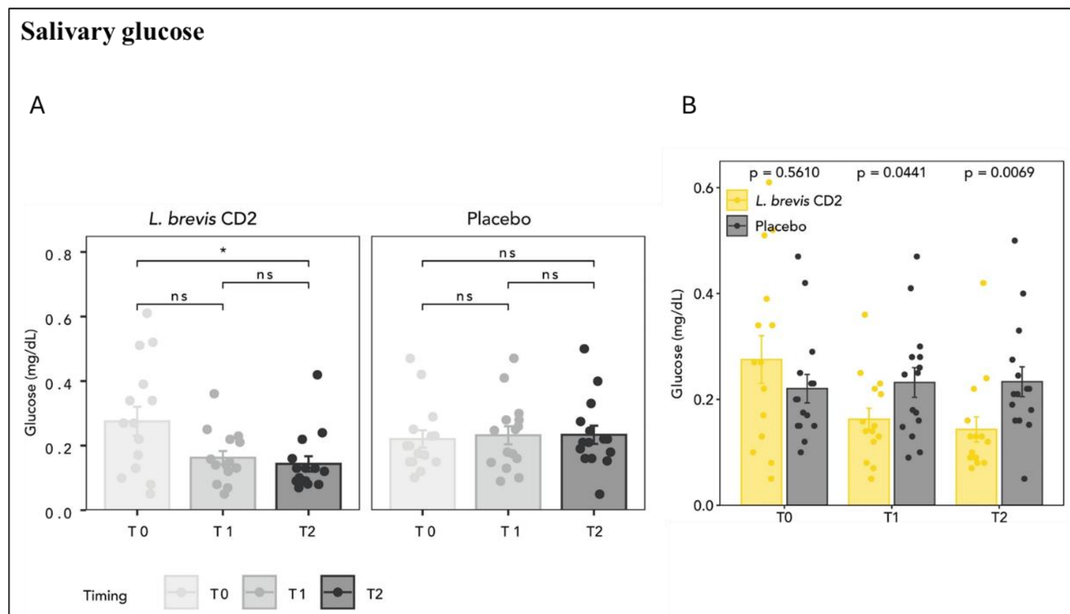


Figure 15. Evaluation of salivary glucose in the *L. brevis* CD2 and placebo groups at T0, T1, and T2. Salivary glucose expressed as mean ± SE. ns = non-significant, *$p<0.05$, and **$p<0.01$.

In addition, the probiotic intervention led to a significant reduction in salivary D-lactate levels after four weeks of treatment ($p<0.01$), and this effect persisted at T2 ($p<0.01$) (**Figure 16A**). No significant changes were observed in the placebo group across time points. Between-group comparisons confirmed that D-lactate levels were significantly lower in the probiotic group than in the placebo group both at the end of treatment and after the washout ($p<0.01$) (**Figure 16B**).

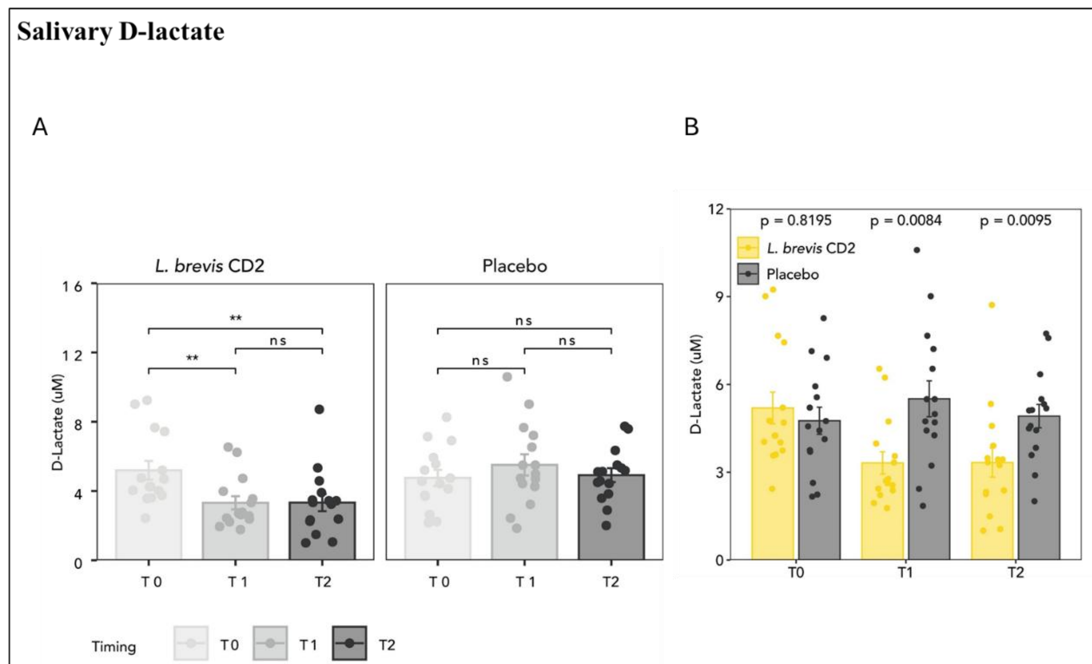


Figure 16. Evaluation of salivary D-lactate in the *L. brevis* CD2 and placebo groups at T0, T1, and T2. Salivary D-lactate expressed as mean ± SE. ns = non-significant, **p<0.05*, and ***p<0.01*.

The intake of *L. brevis* CD2 resulted in a highly significant increase (*p<0.01*) in salivary sIgA levels at T1 compared to T0 (**Figure 17A**), and the effect was supported even at T2, thus maintaining significantly higher values than T0 (*p<0.01*). No significant changes were registered in the placebo group at post-treatment and washout compared to baseline. 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*) (**Figure 17B**).

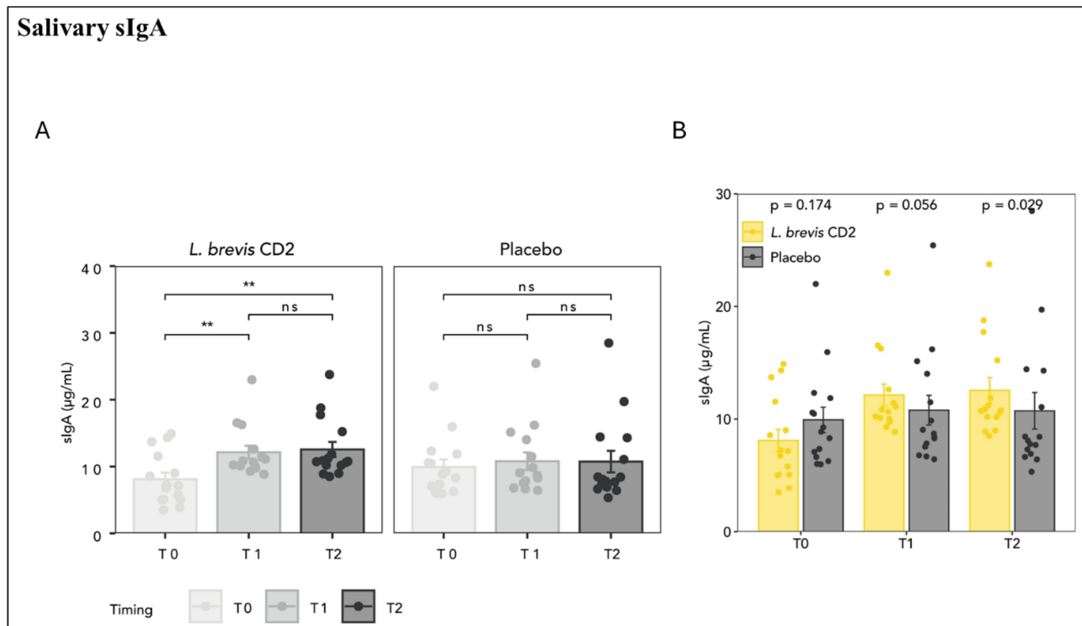


Figure 17. Evaluation of sIgA levels in the *L. brevis* CD2 and placebo groups at T0, T1, and T2. sIgA values (A and B) expressed as mean ± SE. ns = non-significant, *$p<0.05$; **$p<0.01$.

5.5. Effects of L. brevis CD2-Containing Lozenges on Oral Nitrate-Reducing Capacity

The four-week treatment with *L. brevis* CD2-containing lozenges led to a statistically significant enhancement in oral nitrate-reducing capacity compared to baseline ($p<0.005$) (**Figure 18**). This elevated activity persisted even after the washout period, compared with baseline, although the difference was no longer statistically significant (**Figure 18A**). In contrast, the placebo group did not exhibit any significant changes in nitrate-reducing capacity either post-treatment or following the washout phase. When comparing the two groups, the *L. brevis* CD2-treated subjects exhibited a significantly greater nitrate-reducing potential both immediately after the intervention and after the washout period, with statistical significance reached only at T1 ($p<0.01$) (**Figure 18B**).

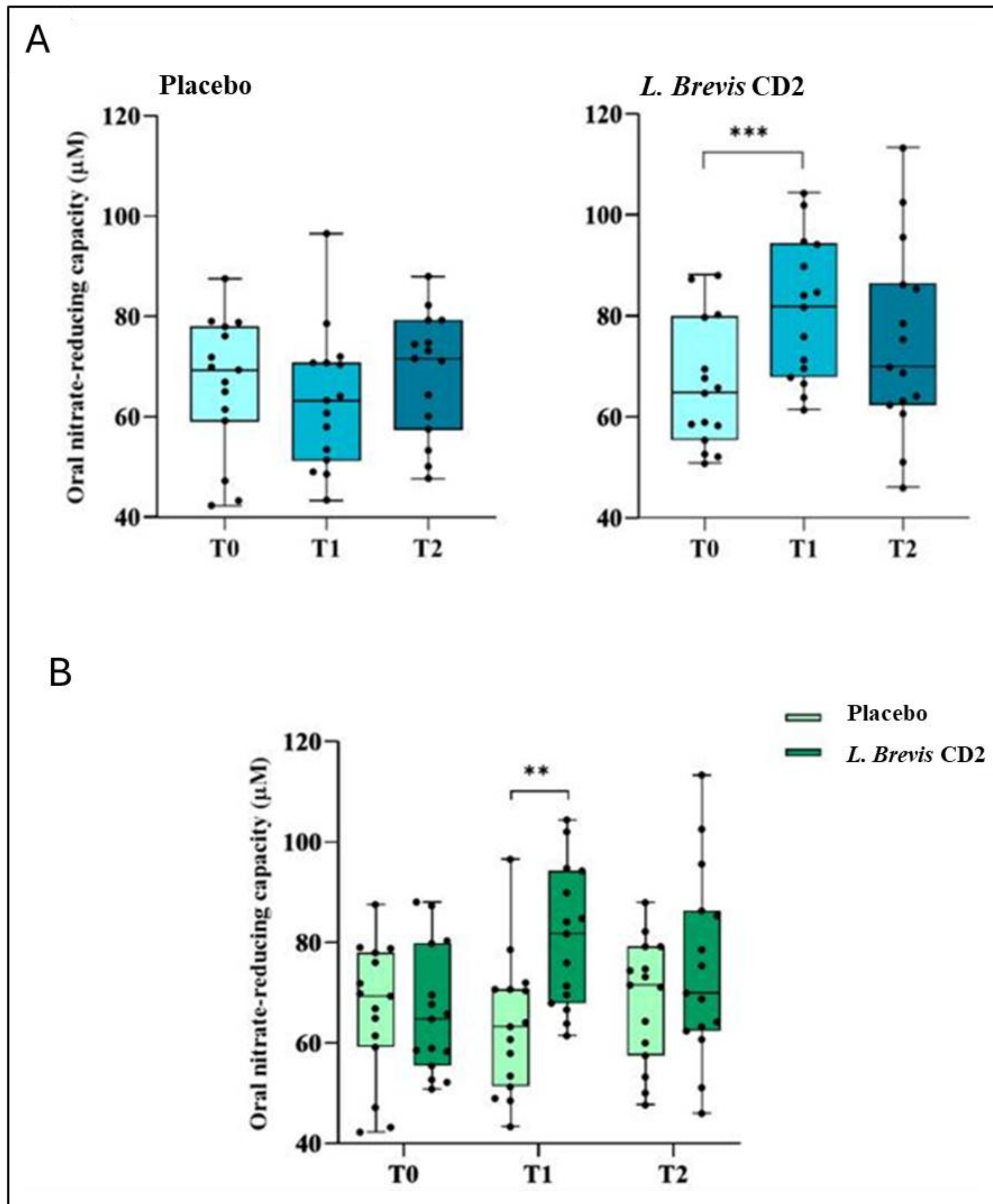


Figure 18. Evaluation of oral nitrate-reducing capacity assessed by nitrite levels (µM) in the placebo and *L. brevis* CD2 groups at T0, T1, and T2 (Panel (A)) (*** $p<0.005$). Panel (B) shows the differences between placebo and probiotic groups at different times (** $p<0.01$). Boxplots show the median and interquartile range (IQR, 25th–75th percentile).

5.6. In Vitro Assessment of Nitrate-Reducing Activity of L. brevis CD2 Lysate

To support the *in vivo* findings on the nitrate-reducing capacity of *L. brevis* CD2-containing lozenges, the enzymatic activity of the probiotic lysate was

evaluated *in vitro* (**Figure 19**). *L. brevis* CD2 lysate demonstrated a concentration-dependent nitrate-reducing activity *in vitro*, as illustrated in **Figure 19A**. The activity, measured in μM, was closely correlated with the probiotic protein concentration introduced into the reaction system, indicating a proportional increase in enzymatic activity. Beyond a concentration of 1600 μg protein/mL, nitrite production remained relatively stable, leading to the selection of this concentration for subsequent experiments involving salivary samples. The use of nitrate reductase as an internal positive control yielded a nitrite concentration of 133.14 ± 2.74 μM when 8 mM nitrate was used as the substrate, a result comparable to that obtained with higher probiotic concentrations. The inset in **Figure 19A** summarizes the enzymatic activity data expressed in units (μmol of nitrite produced per minute). Furthermore, the reduction of nitrates and the corresponding generation of nitrites were dependent on the concentration of the nitrate substrate, as shown in **Figure 19B**. This observation reinforces the specificity of the enzymatic reaction, as confirmed by the absence of nitrite production in the absence of nitrate.

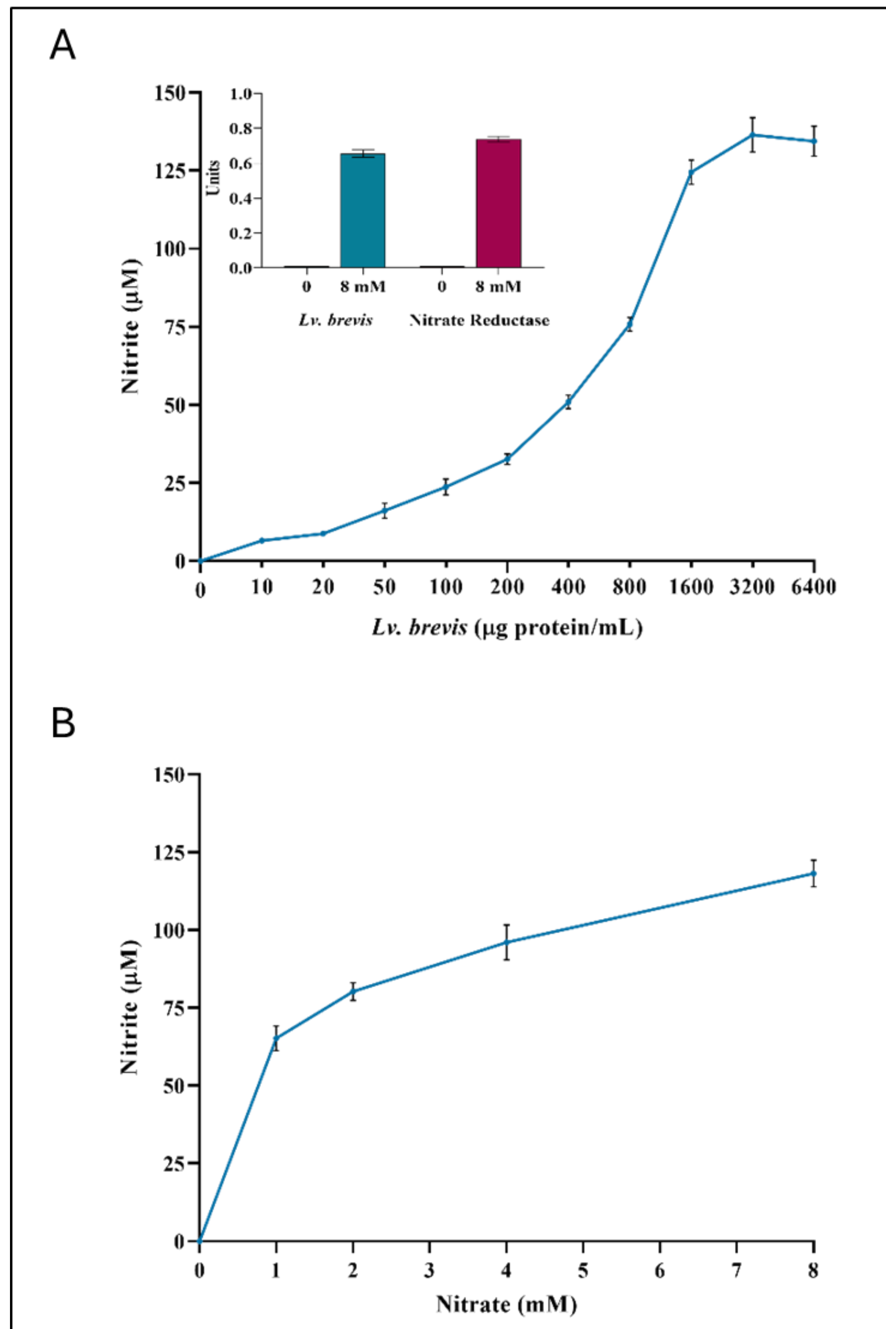


Figure 19. Nitrate reductase activity of *L. brevis* CD2 lysate. (A) Probiotic lysate was incubated at various concentrations (µg protein/mL) with an 8 mM nitrate solution for 3 h at 37 °C. The nitrite concentration in the supernatant was measured using the Griess reaction. The results are expressed as mean ± SE of three independent experiments, each performed in triplicate. The inset summarizes the activity of nitrate reductase, used as an internal positive control, compared with that of the probiotic at 1600 µg protein/mL. (B) The nitrate reductase activity of *L. brevis* lysate, at a concentration of 1600 µg protein/mL, was assessed using sodium nitrate concentrations ranging from 1 to 8 mM. The values are expressed as mean ± SE of three independent experiments performed in triplicate.

5.7. Ex Vivo Assessment of Nitrate-Reducing Activity of L. brevis CD2 Lysate in Human Salivary Samples

To evaluate the enzymatic activity of *L. brevis* CD2 lysate in a biologically relevant context, *ex vivo* assays were conducted using saliva samples collected from six trial participants. Baseline nitrate-reducing activity, determined by measuring nitrite concentrations via the Griess reaction, ranged from 7.69 to 25.4 µM across samples (**Figure 20**). As expected, the addition of an exogenous nitrate solution (8 mM) significantly enhanced salivary nitrate reductase activity across all samples, thereby promoting nitrate reduction. Notably, the addition of *L. brevis* CD2 lysate resulted in a substantial increase in nitrate reduction across all salivary samples compared with untreated controls ($p<0.05$), demonstrating its intrinsic enzymatic activity. The effect of *L. brevis* CD2 was comparable to that of the exogenous nitrate alone. Moreover, the combined application of the probiotic lysate and the nitrate solution resulted in significantly higher nitrite production than either treatment administered individually ($p<0.01$).

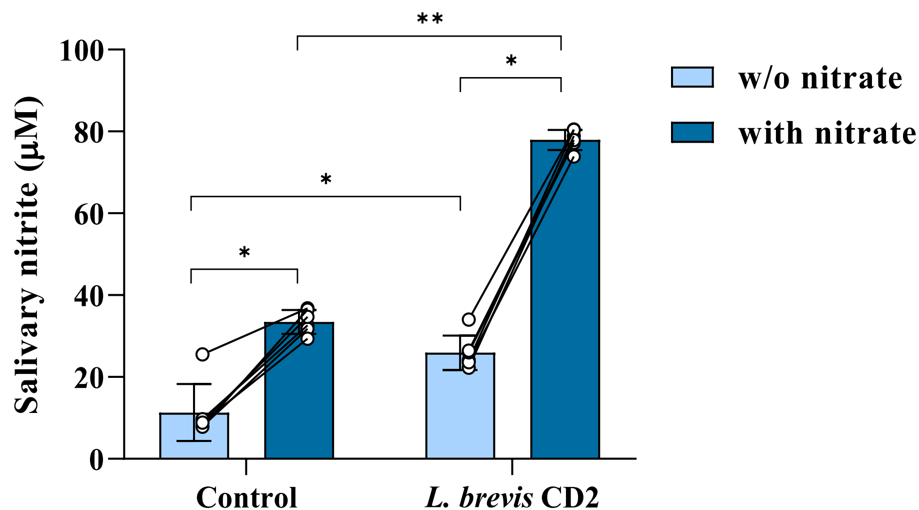


Figure 20. Nitrate reduction activity of *L. brevis* CD2 lysate in saliva samples from trial participants. The bar graphs represent the concentration of nitrite in saliva samples that were incubated *ex vivo* for 3 h at 37 °C without (control) or with nitrate solution (8 mM). Where indicated, the saliva samples were treated with *L. brevis* CD2 lysate (1600 µg protein/mL) with or without the nitrate solution. The circles represent the individual data, while the connecting lines show the changes in each sample with or without nitrate. The values are expressed as mean ± SD of one representative experiment performed in triplicate from three independent experiments. Within-group comparisons were performed using a

two-sided paired Wilcoxon signed-rank test, and between-group comparisons were performed using a two-sided Mann–Whitney test (* $p<0.05$; ** $p<0.01$).

5.8. Ex Vivo Evaluation of L. brevis CD2 Lysate Effects on Salivary pH and Buffering Capacity in Salivary Samples with and without Exogenous Nitrate

As expected, untreated control samples showed no significant variation in salivary pH after 3 hours of incubation at 37 °C (T1) compared to baseline values (T0), as shown in **Figure 21A**. Conversely, incubation with *L. brevis* CD2 resulted in a significant increase in salivary pH relative to T0, both in the presence and absence of exogenous nitrates ($p<0.05$) (**Figure 21A**). When comparing groups, saliva samples treated with *L. brevis* CD2 exhibited significantly higher pH values than their respective untreated controls ($p<0.05$). Notably, the combined administration of the probiotic and exogenous nitrate resulted in a greater increase in pH than the corresponding controls ($p<0.01$).

Regarding buffering capacity, a 3-hour incubation with *L. brevis* CD2 lysate (T1) led to a significant increase compared to baseline (T0), both in the presence and absence of exogenous nitrate ($p<0.05$) (**Figure 21B**). In contrast, untreated control samples showed no significant change in buffering capacity between T0 and T1 (**Figure 21B**). Although the buffering capacity of saliva samples treated with *L. brevis* CD2 increased compared to untreated controls, this difference did not reach statistical significance. However, the combined treatment with a probiotic and exogenous nitrate produced a more marked increase in buffering capacity than the respective control group ($p<0.05$).

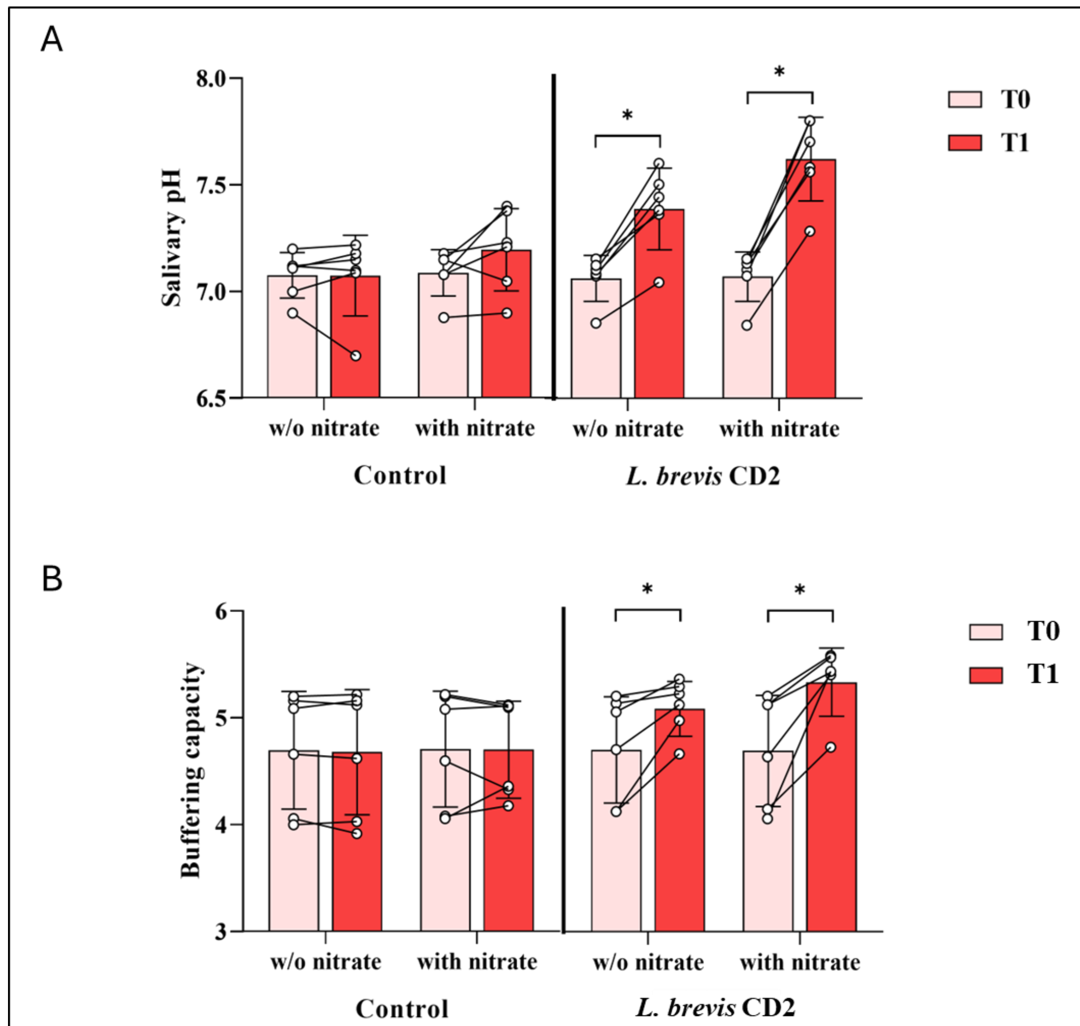


Figure 21. Effect of *L. brevis* CD2 lysate on pH levels of salivary samples at T0 and after 3-hour incubation (T1), both with and without the addition of exogenous nitrates (8 mM) (Panel (A)). Effect of *L. brevis* CD2 on buffering capacity of salivary samples at T0 and after 3-hour incubation (T1) in the presence or absence of exogenous nitrates (8 mM) (Panel (B)). The circles represent the individual data, while the connecting lines show the changes in each sample before (T0) and after (T1) incubation. All the values are expressed as mean ± SD from one representative experiment performed in triplicate of three independent experiments. Within-group comparisons were performed using a two-sided paired Wilcoxon signed-rank test and between-group comparisons using a two-sided Mann–Whitney test (* $p<0.05$).

5.9. Ex vivo Effect of L. brevis CD2 lysate on D-Lactate Levels in Human Salivary Samples

Given the established link between lactate metabolism and nitrite production, in which lactate serves as an electron donor in the nitrate reduction pathway, D-lactate

concentrations were measured in *ex vivo* human saliva samples. Samples were analysed at baseline (T0) and after a 3-hour incubation period (T1), with or without the addition of *L. brevis* CD2 lysate at a concentration of 1600 µg protein/mL. The results demonstrated a significant reduction in D-lactate levels following treatment with the probiotic lysate, compared to untreated controls (*p<0.01*) (**Figure 22**), indicating a relevant modulatory effect of *L. brevis* on salivary lactate metabolism.

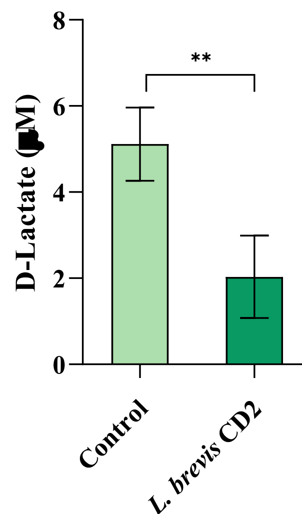


Figure 22. Effect of *L. brevis* CD2 lysate on D-lactate levels in human salivary samples after 3-hour incubation. The values are expressed as mean ± SD of one representative experiment performed in triplicate from three independent experiments. Between-group comparisons were performed using a two-sided Mann–Whitney test (** *p<0.01*).

The heatmaps in **Figure 23** show the values of all evaluated salivary parameters for individual samples, comparing untreated controls with those treated with *L. brevis* CD2 lysate at T1. Darker shades indicate higher values, while lighter shades represent lower values. *Z*-score normalization highlights the individual response to probiotic treatment, revealing a consistent improvement across all measured salivary parameters compared to untreated samples.

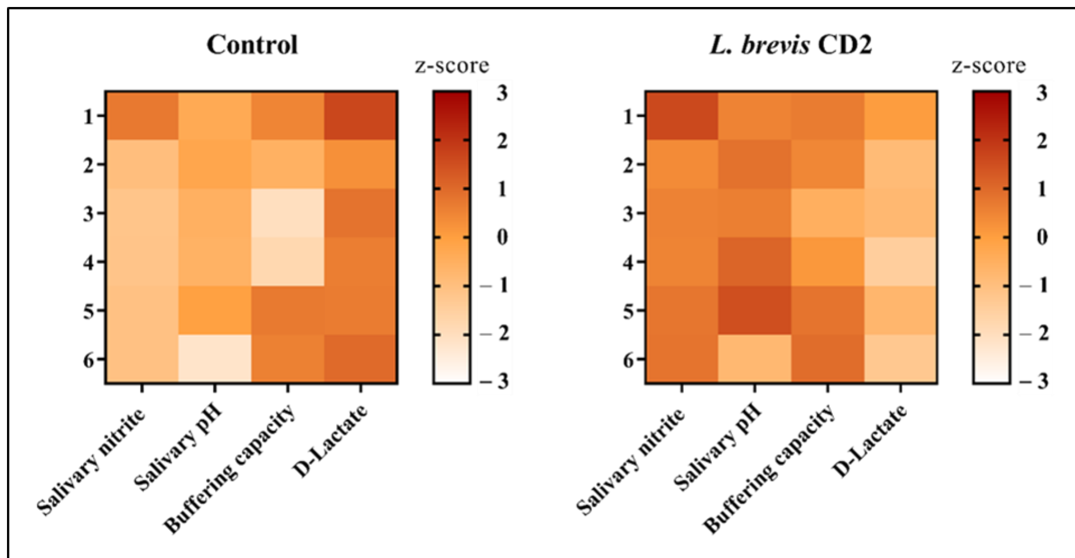


Figure 23. Heatmap illustrating the z-scores of the parameters evaluated in saliva samples incubated with or without *L. brevis* CD2 lysate for 3 h. Darker shades indicate higher values, while lighter shades represent lower values. The left heatmap displays the untreated saliva samples (control), while the right heatmap represents the saliva samples treated with *L. brevis* CD2 lysate.

5.10. Effects of L. brevis CD2-Containing Lozenges on sAA Levels

The *in vivo* analysis of sAA levels was performed using a colorimetric assay, as previously described. In the *L. brevis* group, sAA levels showed a significant reduction at T1 compared to baseline (T0) ($p<0.01$). Although sAA levels remained lower at T2 than at T0, the difference was no longer statistically significant. In contrast, the placebo group exhibited no significant changes in sAA levels at either T1 or T2 relative to baseline (**Figure 24A**). At baseline, both groups presented comparable sAA values. However, a statistically significant difference emerged between the groups at T1, with lower sAA levels in the *L. brevis* group ($p<0.05$). This trend persisted at T2, although the intergroup difference did not reach statistical significance (**Figure 24B**).

To further confirm the ability of the probiotic-containing lozenge to inhibit enzyme activity, a probiotic lysate was obtained from *L. brevis* lozenges as described in the materials and methods section, and an *in vitro* assay of enzymatic inhibition was conducted. Briefly, commercial sAA was incubated with bacterial lysate from *L. brevis* lozenges at varying concentrations, and sAA activity was evaluated using a colorimetric kit. The results shown in Figure 3C indicated that the lysate from *L. brevis* lozenge could reduce

sAA activity in a dose-dependent manner; concentrations of 0.25, 0.5, and 1 mg/ml of bacterial protein resulted in reductions of approximately 25%, 44%, and 53%, respectively. This reduction was significant at all tested concentrations.

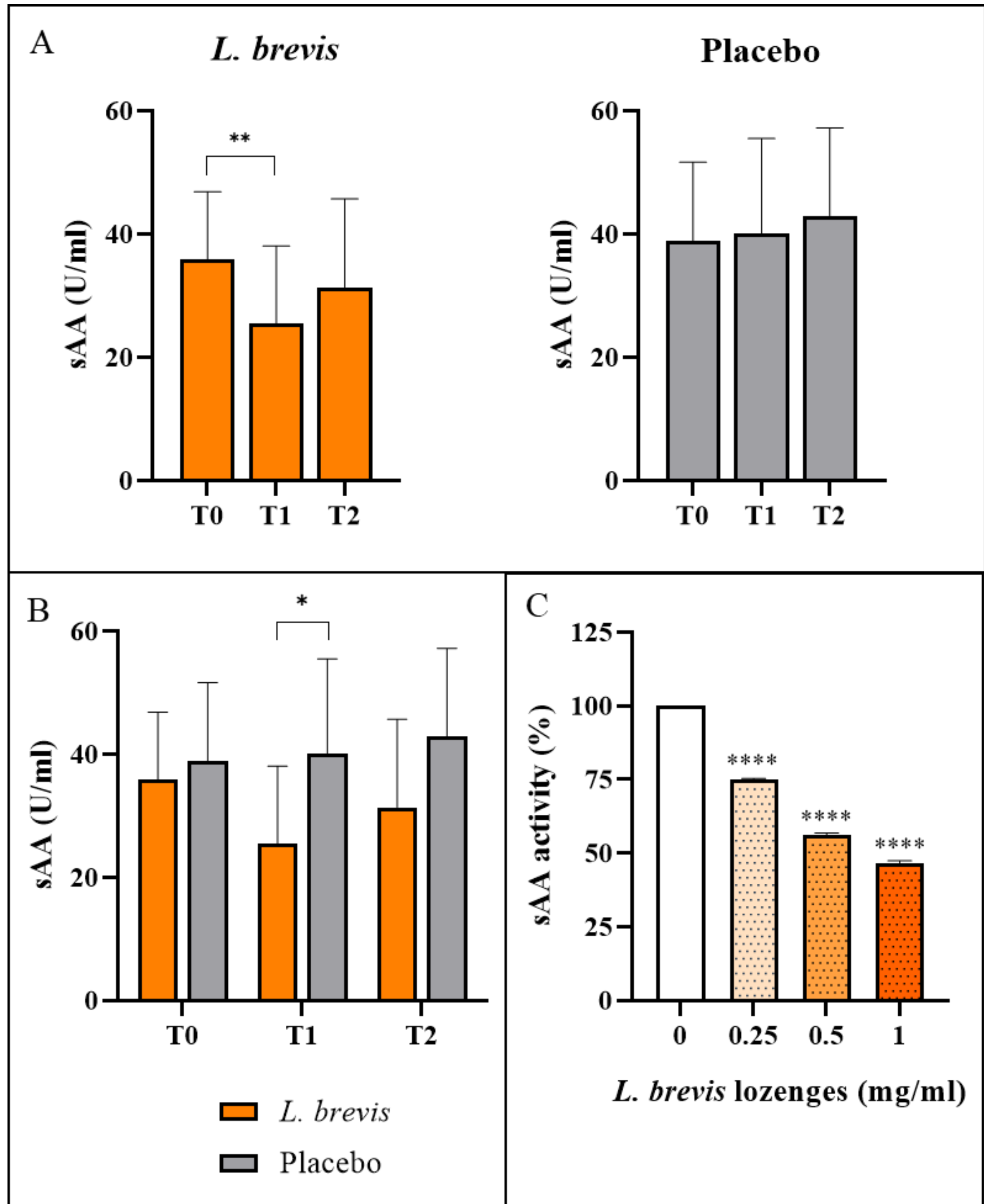


Figure 24. Effect of *L. brevis* CD2 lozenges on sAA activity *in vivo* and *in vitro*. (A, B) sAA activity (U/ml) was measured in saliva samples from the *L. brevis* and Placebo groups using a commercial kit, as reported in the Materials and Methods section and shown as mean ± SD. (C) sAA (60 mU/ml) was incubated with increasing protein concentrations of *L. brevis* lozenges lysate; then, the enzymatic

activity was evaluated. Data expressed as mean ± SEM are from three independent experiments performed in duplicate. (*$p<0.05$, **$p<0.01$, ****$p<0.0001$).

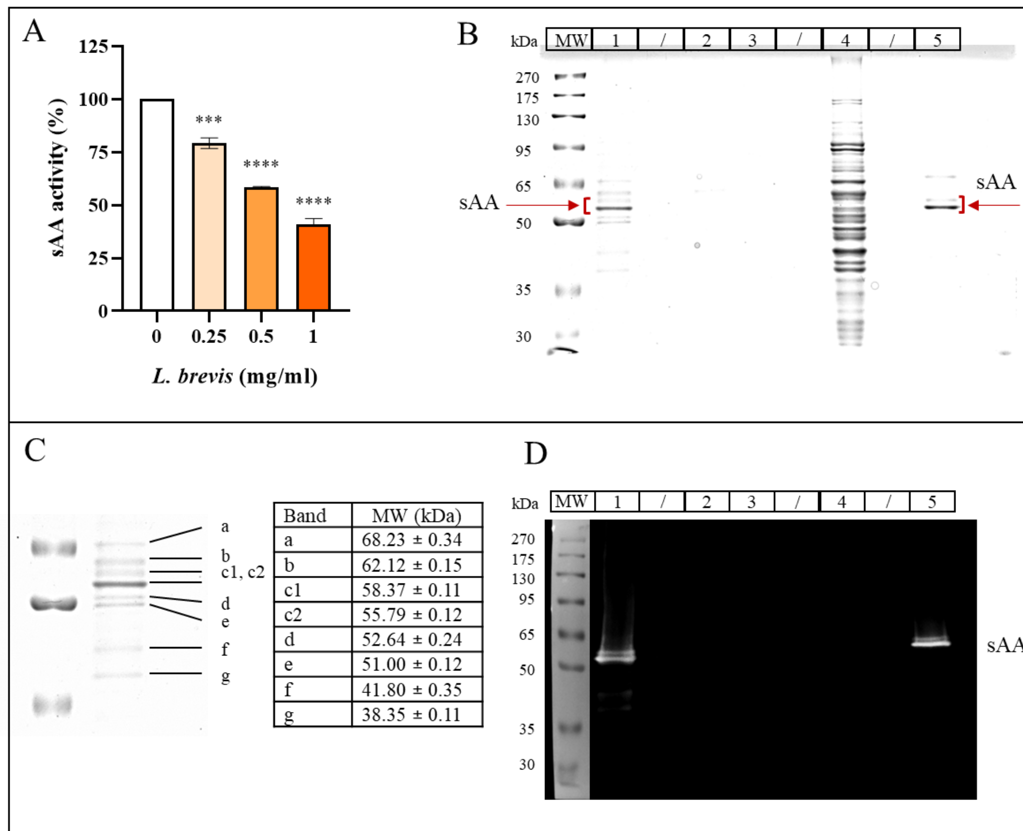
5.11. In Vitro Effects of L. brevis CD2 lysate on sAA levels

To further investigate the inhibitory potential of *L. brevis* CD2 on sAA activity, an *in vitro* assay was performed using a lysate derived from its lyophilized form. As shown in **Figure 25A**, the lysate significantly reduced sAA activity in a dose-dependent manner, with protein concentrations of 0.25, 0.5, and 1 mg/mL resulting in approximately 20%, 41%, and 59% inhibition, respectively.

To explore the mechanism underlying this inhibitory effect, the presence of soluble components capable of interacting with sAA was examined. For this purpose, an amylase-binding assay [Nikitkova et al, 2012] was conducted to assess whether the lysate contained proteins that could bind to sAA and potentially mediate its inhibition. The lysate was incubated with sAA, while control samples included lysate and sAA incubated separately under identical conditions.

SDS-PAGE analysis of the precipitate obtained from the co-incubation (**Figure 25B**) revealed bands corresponding to sAA, as confirmed by comparison with lane 5, which contained sAA alone as a positive control. Additionally, six distinct protein bands were detected. Their apparent molecular weights (MW), estimated by Coomassie staining and analyzed with Alliance Uvitec software (see Materials and Methods), are shown in **Figure 25C**. Bands c1 and c2 exhibited MWs of 59.02 ± 0.33 kDa and 56.33 ± 0.28 kDa, respectively, consistent with glycosylated and non-glycosylated forms of sAA [Contreras-Aguilar et al, 2021]. Additional bands in the precipitate ranged from approximately 38 to 68 kDa.

Notably, no bands were observed in the control lanes (2 and 3), indicating that the precipitated proteins resulted specifically from the interaction between sAA and components of the *L. brevis* lysate. To confirm the identity of the sAA bands, proteins from a duplicate gel were transferred to a nitrocellulose membrane and probed with an anti-sAA antibody. The results, presented in **Figure 25D**, verified the presence of sAA in the precipitate formed during co-incubation with the lysate.

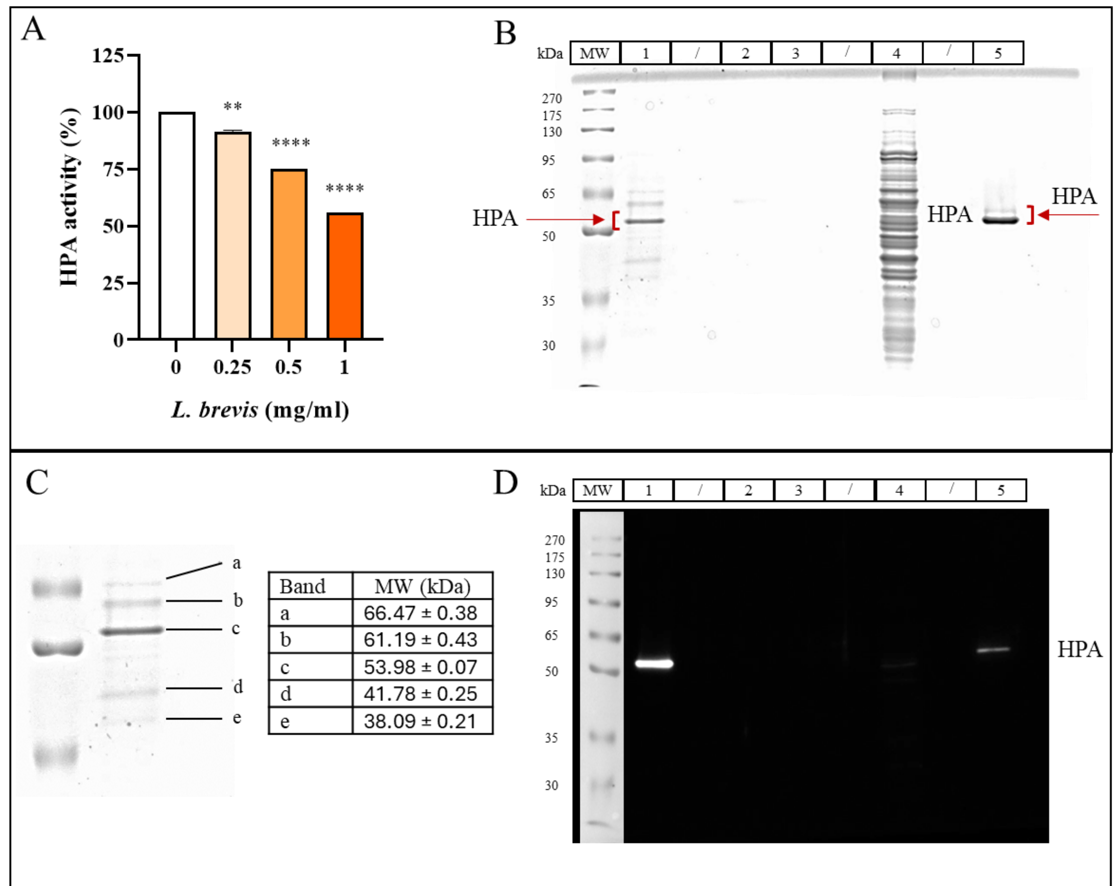


Band	MW (kDa)
a	68.23 ± 0.34
b	62.12 ± 0.15
c1	58.37 ± 0.11
c2	55.79 ± 0.12
d	52.64 ± 0.24
e	51.00 ± 0.12
f	41.80 ± 0.35
g	38.35 ± 0.11

Figure 25. Effect of *L. brevis* CD2 lysate on sAA. (A) sAA (60 mU/ml) was incubated with increasing concentrations of *L. brevis* lysate, and enzymatic activity was subsequently assessed using a colorimetric amylase activity assay. Data are expressed as mean ± SEM from three independent experiments, each performed in duplicate. (B, D) Representative SDS-PAGE gel and Western blot (WB) membrane from the amylase binding assay. sAA (100 µg/ml) was co-incubated with *L. brevis* lysate proteins (1 mg/ml) for 2 hours at 25°C. Control samples included sAA and *L. brevis* lysate incubated separately under the same conditions. After incubation, all samples were resolved by SDS-PAGE. Gels were either stained with Coomassie Blue (B) or transferred to nitrocellulose membranes for sAA detection using an anti-sAA antibody (D). Lane assignments are as follows: Lane 1: sAA co-incubated with *L. brevis* lysate; Lane 2: *L. brevis* lysate incubated alone; Lane 3: sAA incubated alone; Lane 4: non-incubated *L. brevis* CD2 lysate control (25 µg); Lane 5: non-incubated sAA control (2 µg for SDS-PAGE, 1 µg for WB). For WB imaging, molecular weight markers were captured separately in bright field before chemiluminescence and digitally aligned with the membrane image for accurate visualization. (C) A zoomed-in view of lane 1 is shown, accompanied by a table reporting the molecular weight analysis performed using Alliance Uvitec software, as described in the Materials and Methods section. Results are expressed as mean ± SEM from three independent experiments. Statistical significance is indicated as follows: ****p<0.001*, *****p<0.0001* compared to sAA not incubated with *L. brevis* lysate.

5.12. In Vitro Effects of L. brevis CD2 Lysate on HPA Levels

Given the high degree of amino acid sequence homology between sAA and HPA (97% sequence identity [Visvanathan et al., 2024]), the potential inhibitory effect of *L. brevis* CD2 lysate on HPA was investigated. The results, presented in **Figure 26**, confirmed a dose-dependent inhibition of HPA activity by the probiotic lysate. Under the experimental conditions, activity reductions of approximately 9%, 25%, and 44% were observed at protein concentrations of 0.25, 0.5, and 1 mg/mL, respectively. Additionally, proteins from *L. brevis* CD2 co-precipitated with HPA, displaying a partially overlapping band pattern with that observed in the sAA assay (**Figure 26B)**. Molecular weight analysis identified bands labeled a, b, c, and d in **Figure 26C**, which correspond to bands a, b, f, and g in Figure 25C, although two bands present in the sAA gel were absent in the HPA assay. WB analysis further confirmed the identity of HPA in the precipitated complex.



Band	MW (kDa)
a	66.47 ± 0.38
b	61.19 ± 0.43
c	53.98 ± 0.07
d	41.78 ± 0.25
e	38.09 ± 0.21

Figure 26. Effect of *L. brevis* CD2 lysate on HPA. (A) HPA (15 mU/ml) was incubated with increasing concentrations of *L. brevis* lysate, and its enzymatic activity was subsequently assessed using a colorimetric amylase activity assay. Data are presented as mean ± SEM from three independent experiments, each performed in duplicate. (B, D) Representative SDS-PAGE gel and WB membrane from the amylase binding assay. HPA (100 µg/ml) was co-incubated with *L. brevis* lysate proteins (1 mg/ml) for 2 hours at 25°C under agitation. Control samples included HPA and *L. brevis* lysate incubated separately under identical conditions. Following incubation, all samples were resolved by SDS-PAGE. Gels were either stained with Coomassie Blue (B) or transferred to nitrocellulose membranes for HPA detection using an anti-HPA antibody (D). Lane assignments are as follows: Lane 1: HPA co-incubated with *L. brevis* CD2 lysate; Lane 2: *L. brevis* CD2 lysate incubated alone; Lane 3: HPA incubated alone; Lane 4: non-incubated *L. brevis* CD2 lysate control (25 µg); Lane 5: non-incubated HPA control (2 µg for SDS-PAGE, 1 µg for WB). For WB imaging, molecular weight markers were acquired separately in bright field before chemiluminescence and digitally aligned with the membrane image for accurate visualization. (C) A magnified view of lane 1 is shown, along with a table reporting the molecular weight analysis performed using Alliance Uvitec software, as described in the Materials and Methods section. Results are expressed as mean ± SEM from three independent experiments. (**$p<0.01$, ****$p<0.0001$ vs HPA not incubated with *L. brevis* lysate).

5. DISCUSSION

This thesis explores the therapeutic and preventive potential of the probiotic strain *Levilactobacillus brevis* CD2 (CNCM I-5566) for promoting oral health through an integrated approach combining *in vivo*, *ex vivo*, and *in vitro* investigations. The research is based on a randomized controlled clinical trial designed to evaluate the effects of this probiotic on both clinical and biochemical parameters of oral health. Specifically, a clinical evaluation was conducted in healthy adults who underwent 4 weeks of supplementation with lozenges containing *L. brevis* CD2. The effectiveness of the intervention was assessed by monitoring two key indicators of periodontal health: bleeding on probing (BoP) and plaque index (PI).

The findings demonstrate a significant and sustained impact of *Lactobacillus brevis* CD2 on periodontal health, as evidenced by reductions in both BoP and PI scores. After 4 weeks of treatment, the probiotic group showed a highly significant decrease in BoP from baseline, with values remaining significantly lower even after a 2-week washout period. This suggests a lasting anti-inflammatory effect of *L. brevis* CD2 on gingival tissues. Similarly, PI scores were significantly reduced following the probiotic intervention and persisted after washout, indicating a stable effect on plaque accumulation. Correlation analyses revealed a strong

positive relationship between changes in BoP and PI scores, suggesting that improvements in plaque control may reduce gingival inflammation. However, mediation analysis indicated that the effect of *L. brevis* CD2 on BoP was primarily direct, rather than mediated by changes in PI. The indirect effect was not statistically significant, and the confidence interval included zero, confirming that the probiotic's influence on BoP operates independently of plaque reduction.

These results have important clinical implications. The ability of *L. brevis* CD2 to significantly reduce BoP and PI scores, and to maintain these improvements beyond the treatment period, suggests its potential as a supportive therapy in periodontal care. The direct anti-inflammatory effect on gingival tissues, independent of plaque reduction, highlights its potential in patients with gingivitis or early-stage periodontitis, especially those who struggle with mechanical plaque control.

Moreover, the use of *L. brevis* CD2-containing lozenges could represent a non-invasive, patient-friendly adjunct to conventional oral hygiene practices, potentially improving compliance and outcomes in periodontal therapy. Future studies should explore its long-term effects, mechanisms of action, and applicability across broader patient populations, including those with systemic conditions associated with periodontal inflammation.

A comprehensive salivary analysis was also performed to investigate potential changes in composition and function. The study revealed a notable improvement in salivation rate among subjects treated with *L. brevis* CD2. To date, this is the first evidence demonstrating a positive impact of *L. brevis* CD2 on this critical parameter, which plays a central role in maintaining both oral and systemic health [Govindaraj et al., 2019; Melo et al., 2023]. Previous studies have reported that reduced salivary flow is associated with increased caries activity and various systemic conditions, including Sjögren's syndrome and diabetes. Moreover, certain therapeutic interventions, such as pharmacological treatments or radiation therapy for head and neck cancers, can impair salivary gland function [Carramolino-Cuéllar et al., 2018; Martins et al., 2022; Castelli et al., 2023]. In

contrast, higher salivary flow rates are linked to enhanced buffering capacity, improved remineralization/demineralization balance, increased antimicrobial activity, and strengthened immune responses. These factors collectively contribute to a less favourable environment for the development of cariogenic biofilms [Dodds et al., 2005], a consideration of particular relevance for oral health professionals.

Another objective of the study was to assess whether 4-week treatment with *L. brevis* CD2-containing lozenges could preserve salivary pH, a key factor in maintaining oral cavity health and a significant indicator of caries risk. Low salivary pH promotes the growth of aciduric and acidogenic bacteria, creating conditions detrimental to the protective oral microbiota [Georgios et al., 2015]. The findings highlight the probiotic's ability to maintain physiological pH levels in treated subjects. This effect is likely attributable to the arginolytic properties of the *L. brevis* CD2 strain, which facilitates the production of ammonia ions capable of counteracting acidification and sustaining a salivary pH above 7 [Marquis et al., 1987; Riccia et al., 2007; Nascimento et al., 2009; Abruzzo et al., 2020]. This result is particularly noteworthy given that some probiotics tested *in vitro* have been shown to reduce salivary pH [Singh and Doley, 2016; Faraz et al., 2019; Saha et al., 2023].

Saliva's buffering capacity is essential for stabilizing pH, neutralizing acid exposure, and preventing enamel demineralization and dental caries [Dipalma et al., 2023]. Factors contributing positively to buffering capacity include bicarbonate ions (HCO_3^-), proteins, phosphates, and elevated salivary flow rates [Boteon et al., 2023]. The present study identified a statistically significant improvement in buffering capacity among all subjects treated with *L. brevis* CD2, compared to both baseline and placebo values.

Regarding the impact of probiotic treatment on salivary glucose levels, although statistical significance was not reached, a downward trend was observed following the intake of *L. brevis* CD2 compared to baseline. Considering the established role of salivary glucose as a biomarker for dental caries and its

association with systemic conditions such as diabetes, these findings may be relevant to the prevention of both oral and systemic diseases [Naing and Mak, 2017; Almusawi et al., 2018; Manjushree et al., 2022].

More notably, *L. brevis* CD2 treatment resulted in a significant and sustained reduction in salivary D-lactate levels compared to baseline and placebo. This effect persisted even after the washout period, representing the first evidence of a probiotic's ability to modulate baseline salivary D-lactate concentrations. Since D-lactate is a stereoisomer of lactic acid produced during bacterial carbohydrate metabolism, primarily from glucose [Liang et al., 2018], its reduction may be linked to the probiotic's influence on glucose metabolism. D-lactate accumulation has been implicated in inflammatory bowel disease and metabolic disorders, both of which share associations with oral dysbiosis and dental caries [Majbauddin et al., 2019; Haznedaroglu and Polat, 2023; Morace et al., 2024; Remund et al., 2023]. Thus, *L. brevis* CD2's ability to lower both salivary glucose and D-lactate levels may contribute to its cariostatic and anti-inflammatory potential.

Probiotics are also known to influence the composition of saliva, including mucins and salivary immunoglobulin levels, which, in turn, affect the quantity and quality of saliva secreted. Among these components, sIgA plays a central role in local immunity and host defence. sIgA contributes to oral health by opsonizing and agglutinating bacteria, thereby preventing their adhesion to the mucosal epithelium of the gastrointestinal tract, including the oral cavity [Marcotte and Lavoie, 1998; Ranadheer et al., 2011; Herich, 2017]. Notably, a significant and sustained increase in sIgA levels was observed following the four-week *L. brevis* CD2 treatment, with the effect persisting after the washout period. Although mean sIgA levels were higher in the probiotic group than in the placebo group, statistical significance was achieved only at follow-up, suggesting a delayed but sustained immunomodulatory effect.

The promising outcomes of probiotic use in preventing major oral diseases, such as periodontitis and dental caries, underscore the need for further research and development in this field. The present findings suggest that *L. brevis* CD2

may exert a notable preventive effect on oral disease maintenance, with significant improvements in both clinical parameters and salivary biomarkers. The clinical relevance of consuming dairy products containing this probiotic highlights its potential as a supportive strategy for preventing dental caries and other oral conditions. These results align with growing evidence indicating that specific *Lactobacillus* strains can enhance or preserve oral cavity health [Wang et al., 2022; Homayouni et al., 2023]. Certain *Lactobacillus* strains have been shown to release factors with immunomodulatory and anti-inflammatory properties [Wang et al., 2022; Homayouni et al., 2023]. These bioactive compounds contribute to maintaining a balanced microbial community through competitive and antibacterial interactions with pathogenic bacteria. Evidence supports the ability of specific *Lactobacillus* strains to prevent periodontal disease by inhibiting dental plaque formation [Tobita et al., 2018]. This protective effect has been linked to the production of lipase enzymes that degrade pathogenic biofilms.

Additionally, the beneficial impact of selected *Lactobacillus* strains on periodontal tissues has been associated with enhanced proteolytic resistance of connexins, such as E-cadherin and β-catenin, in epithelial cells exposed to pathogens like *P. gingivalis* [Abe-Yutori et al., 2017; Yamada et al., 2018]. Metabolic products released by these strains, including exopolysaccharides, have also demonstrated strong antioxidant properties [Mendi and Aslim, 2014; Mahdhi et al., 2017], and some species exhibit antioxidative functions by upregulating antioxidant enzymes such as heme oxygenase 1 in gingival epithelial cells [Yamada et al., 2018]. Various biochemical and molecular mechanisms have been identified to explain how *Lactobacillus* strains modulate immune-inflammatory responses [Wang et al., 2022; Homayouni et al., 2023]. These include the suppression of NOS2, MMPs, and pro-inflammatory cytokines [Riccia et al., 2007]. Notably, the high levels of ADI present in *L. brevis* may competitively inhibit NOS, thereby contributing to the suppression of inflammatory processes associated with periodontitis [Riccia et al., 2007].

An important extension of the present study concerns the nitrate-reducing potential of *L. brevis* CD2, which was evaluated through *in vivo*, *in vitro*, and *ex vivo* approaches. A four-week treatment with *L. brevis* CD2 lozenges significantly enhanced oral nitrate-reducing capacity *in vivo*. Although the observed benefits persisted beyond the treatment period, statistical significance was not maintained, potentially indicating that the duration of administration was insufficient or that continuous probiotic use may be required to sustain the effects. Importantly, the placebo group showed no changes at any time point, confirming the specificity of the probiotic's effect. Between-group comparisons revealed a significant increase in nitrate-reducing activity in the *L. brevis* CD2 group at T1, further supporting the probiotic's role in modulating microbial nitrate metabolism. These *in vivo* findings are mechanistically supported by *in vitro* and *ex vivo* data.

In vitro assays demonstrated a significant, concentration-dependent intrinsic nitrate reductase activity of *L. brevis* CD2 lysate. This enzymatic activity was also evident when the probiotic was added to saliva samples from six trial participants and was further enhanced by the presence of exogenous nitrates. These results suggest that *L. brevis* CD2 may contribute to increased production and bioavailability of nitrate-derived compounds.

A further objective of the study was to evaluate the *ex vivo* impact of *L. brevis* CD2 lysate on salivary pH. Although baseline pH values of the saliva samples were within the normal range, treatment with the probiotic led to a significant increase in pH across all samples. This suggests that the nitrate-reducing activity of *L. brevis* CD2 contributes to an increase in salivary pH, thereby inhibiting the acidogenic activity of cariogenic bacteria [Doel et al., 2004]. Moreover, the combination of *L. brevis* CD2 with exogenous nitrate produced a more pronounced increase in salivary pH than either treatment alone, reinforcing the role of nitrate reduction in modulating oral pH. The buffering capacity of saliva also showed significant improvement following probiotic treatment compared to untreated samples. This parameter is essential for neutralizing acid exposure, maintaining pH stability, and preventing enamel demineralization and dental

caries [Dipalma et al., 2023]. These findings are consistent with previous data indicating that *L. brevis* CD2 protects dental enamel from demineralizing agents and enhances enamel resistance *in vitro* [Altamura et al., 2024]. Therefore, the nitrate-reducing activity of *L. brevis* CD2 may represent an additional mechanism for counteracting oral acidification and preventing oral diseases such as dental caries.

The current study explores an intriguing relationship between the nitrate-reducing capacity of *L. brevis* CD2 and increases in salivary pH and buffering potential. This dual action may contribute to a more stable and protective oral environment. Specifically, the nitrate reduction pathway appears to elevate salivary pH by utilizing lactate and other electron donors to convert nitrate into nitrite, thereby limiting the accumulation of acidic byproducts. This mechanism not only suppresses the proliferation of acidogenic bacteria but also promotes conditions favourable to beneficial oral microbes [Rosier et al., 2018; Marsh, 2018]. Additionally, the oral microbiome comprises interconnected metabolic networks that may interact with nitrate reduction. For example, the ADI system and urea catabolism release ammonia, which neutralizes acids and increases oral pH [Burne and Marquis, 2000; Nascimento et al., 2019]. It is plausible that *L. brevis* CD2, through its nitrate-reducing activity, may act synergistically with these acid-neutralizing pathways, thereby collectively enhancing the buffering capacity of the oral environment. Further investigations are needed to determine whether co-administration of specific LAB strains, combined with diets rich in arginine or nitrates, may exert additive or complementary effects in stabilizing salivary pH and supporting oral homeostasis.

An additional focus of the study was the effect of *L. brevis* CD2 on salivary D-lactate levels. While most research on LAB emphasizes L-lactate metabolism, D-lactate has distinct clinical and inflammatory implications [Remund et al., 2023]. Accumulation of D-lactate in the oral and gastrointestinal environments has been linked to dysbiosis and inflammatory responses, although the underlying mechanisms remain under investigation [Hove et al., 1999; Levitt and Levitt,

2020; Remund et al., 2023]. The current findings demonstrate a significant reduction in salivary D-lactate levels following treatment with *L. brevis* CD2 lysate compared with untreated controls. These results are consistent with *in vivo* data mentioned above, showing that one month of treatment with lozenges containing the live probiotic led to a significant and sustained decrease in D-lactate levels, at the end of treatment and during a two-week follow-up. This represents the first evidence of a probiotic's ability to modulate baseline salivary D-lactate levels. The observed reduction in D-lactate suggests a potentially relevant mechanism through which *L. brevis* CD2 may contribute to lowering inflammatory or cariogenic risk. Additionally, the link between lactate consumption and nitrate reduction, previously described in specific oral bacterial strains [Rosier et al., 2020; Wicaksono et al., 2020], appears to apply to *L. brevis* CD2, further supporting its role in modulating oral biochemical pathways.

Further investigation is needed to understand how *L. brevis* CD2 influences the composition and function of the oral microbiome. Ongoing research from the associated clinical trial is currently focused on key nitrate-reducing genera, including *Neisseria*, *Rothia*, *Actinomyces*, and *Kingella* [Rosier et al., 2020; Rosier et al., 2022]. The nitrate–nitrite–nitric oxide pathway, which depends on oral microbial activity, has been linked to various cardiovascular and metabolic benefits when stimulated by dietary nitrate intake [Lundberg et al., 2008; Lundberg et al., 2018]. These benefits include reductions in blood pressure, improved endothelial function, reversal of metabolic syndrome, antidiabetic effects, and enhanced physical performance under certain conditions [Kapil et al., 2013; Lundberg et al., 2018; Morou-Bermúdez et al., 2022]. The findings of this study support the potential of *L. brevis* CD2 as a safe and effective probiotic candidate for maintaining or restoring healthy nitrate-reducing activity in the oral cavity, applicable in both preventive and therapeutic contexts. However, the safety profile of nitrate-reducing bacterial strains must be carefully considered. Some species, such as *Staphylococcus aureus*, possess nitrate-reducing capabilities but also exhibit pathogenic traits [Li et al., 2023]. Therefore, further research is

warranted to ensure the safe application of such probiotics in clinical and consumer settings.

Additionally, monitoring for potential nitrosamine formation following the intake of nitrate-reducing probiotics is recommended [Berends et al., 2019]. Several studies have emphasized that combining nitrate-reducing probiotics with a balanced diet rich in dietary nitrates is both safe and effective [Rosier et al., 2020; Milton- Laskibar et al., 2021]. This integrated approach may offer a practical and promising strategy for enhancing NO production and maintaining NO homeostasis, potentially contributing to symptom relief and reducing the incidence and severity of various diseases. As such, targeted use of oral nitrate-reducing probiotics, in conjunction with nitrate-rich nutrition, may represent an innovative avenue for disease prevention and health promotion.

sAA plays a central role in the initial digestion of starch within the oral cavity. It becomes incorporated into dental plaque and serves as a binding site for amylase-binding bacteria, influencing bacterial colonization and potentially contributing to tooth demineralization [Scannapieco et al., 1993]. While some studies suggest that sAA may inhibit bacterial colonization and disrupt carbohydrate components of biofilm matrices, thereby counteracting caries formation, other research highlights its role in promoting biofilm development [Scannapieco et al., 1993; Lahiri et al., 2021]. Specifically, sAA has been shown to bind to amylase-binding proteins on bacterial surfaces, facilitating streptococcal colonization and promoting biofilm formation by plaque-associated bacteria [Nikitkova et al., 2013]. Moreover, starch digestion by sAA increases carbohydrate availability, which supports bacterial growth. This process can enhance streptococcal colonization, stimulate glucan synthesis, and elevate acid production within biofilms, contributing to the onset and progression of dental caries [Bowen and Koo, 2011].

Previous research has demonstrated that various strains of *L. brevis* can inhibit pancreatic α-amylase [Martiz et al., 2023; Sreepathi et al., 2023]. Building on these findings, the present study also evaluated the effect of *L. brevis* CD2 on sAA

levels in healthy individuals. A reduction in sAA levels was observed following four weeks of daily intake of *L. brevis* CD2-containing lozenges. However, this effect diminished after a two-week washout period, suggesting that continuous supplementation may be necessary to maintain the inhibitory effect. It remains possible that longer treatment durations or increased dosages could result in more sustained inhibition beyond the washout phase.

The experimental findings also revealed an additional mechanism through which *L. brevis* CD2 may exert beneficial effects on oral and systemic health, further supporting its multifunctional properties. It is important to consider that xylitol, present in both the placebo and probiotic lozenges, can influence sAA activity and modulate the oral microbiota. Previous studies have shown that xylitol inhibits sAA in a dose-dependent manner [Chukwuma and Islam, 2015; Wölnerhanssen et al., 2025] and can reduce mutans streptococci counts, although it does not significantly alter the overall composition of the oral microbiome [Söderling and Pienihäkkinen, 2020]. Moreover, chewing xylitol-containing gum has been associated with reductions in cariogenic and periodontal bacteria, contributing to the prevention of related oral diseases [Wu et al., 2022]. Despite these known effects, the inclusion of xylitol in both test and control formulations suggests that the observed differences in sAA activity are primarily attributable to the probiotic intervention.

To further investigate the mechanisms underlying the observed reduction in sAA levels, additional *in vitro* experiments were conducted using *L. brevis* CD2 lysate derived from both lozenges and the lyophilized probiotic form. The results strongly support the hypothesis that the *in vivo* effect is attributable to *L. brevis*'s direct enzymatic inhibition. This prompted an evaluation of whether specific proteins in the probiotic might interact with and bind sAA, thereby enhancing its inhibitory effect. Several Streptococcus species bind sAA via amylase-binding proteins, facilitating biofilm formation on dental surfaces [Lahiri et al., 2021]. Previous studies have shown that the addition of sAA to the culture supernatant of these streptococci leads to the precipitation of soluble amylase-binding proteins

[Nikitkova et al., 2012]. A similar approach was applied in the present study to determine whether *L. brevis* CD2 lysate contains molecules capable of precipitating sAA. The co-incubation of sAA with the lysate resulted in the precipitation of both α-amylase and bacterial proteins capable of binding the enzyme. Interestingly, unlike *Streptococcus* strains, which bind sAA without inhibiting its activity [Scannapieco et al., 1990; Douglas et al., 1992; Sethi et al., 2015], *L. brevis* CD2 demonstrated both binding and inhibition. Since starch digestion by sAA provides a carbohydrate-rich substrate that supports bacterial growth, inhibiting α-amylase activity by *L. brevis* may reduce the availability of nutrients for cariogenic bacteria. This mechanism could contribute to the probiotic's protective role in oral health. Future research may explore whether *L. brevis* CD2, through its ability to bind amylase, also participates in the formation of a biofilm with beneficial properties.

Amylases in the human body are primarily produced by the salivary glands and the pancreas. HPA plays a central role in starch digestion and is a key regulator of postprandial blood glucose levels. Acarbose, an FDA-approved drug, inhibits HPA but is associated with gastrointestinal side effects, including flatulence, diarrhoea, and abdominal distension [Xie et al., 2020].

Although sAA and HPA are encoded by distinct genes (AMY1 and AMY2, respectively), they share a high degree of sequence homology and structural similarity. Despite this, their enzymatic activity varies depending on the source of starch [Visvanathan et al., 2024; Peyrot des Gachons and Breslin, 2016]. Recent studies have evaluated the inhibitory effects of over 50 polyphenols on sAA and HPA, revealing that flavonoids exhibit differential inhibition profiles between the two enzymes [Visvanathan et al., 2024]. Based on this evidence, the present study aimed to determine whether *L. brevis* CD2 could interact with and inhibit HPA, in addition to its effects on sAA. *In vitro* experiments confirmed this hypothesis, demonstrating that *L. brevis* CD2 not only inhibits HPA activity but also produces proteins that interact with the enzyme. The interaction profile showed partial overlap with that observed in the sAA experiments. These differences can be

attributed to structural and functional distinctions between the two enzymes. Although sAA and HPA share approximately 97% sequence homology, they differ by 15 amino acid substitutions, which introduce additional polar groups and charged side chains in the salivary isoform. Among these, the substitutions Thr163Ser and Leu196Ile directly affect substrate binding and contribute to differences in specificity toward polysaccharide chain length [Azzopardi et al, 2016].

Previous studies have reported potential hypoglycaemic effects of *L. brevis*, particularly in improving blood glucose levels and insulin resistance in animal models of type 2 diabetes [Chen et al., 2023]. The current findings suggest that these effects may be linked to the probiotic's influence on HPA activity. Additionally, HPA may play a role in intestinal biofilm formation, beyond its established function in starch digestion. For instance, exposure of *Campylobacter jejuni*, a commensal bacterium and a major cause of food-borne gastroenteritis, to pancreatic amylase has been shown to promote biofilm formation in vitro. This exposure enhances bacterial interaction with human epithelial cells, increases virulence in the *Galleria mellonella* infection model, and supports colonization in the chicken ileum [Jowiya et al., 2015].

Therefore, these findings underscore the potential of *L. brevis* CD2 as a promising intervention to improve oral health and suggest a possible role in glycaemic regulation, warranting further investigation. Collectively, the results contribute to a deeper understanding of the anti-cariogenic and potential anti-hyperglycaemic properties of specific probiotics, opening new avenues for research in this field. According to the FAO/WHO 2002 guidelines [FAO and WHO, 2002], any health-related claims regarding probiotics must meet criteria for identity, safety, and demonstrated efficacy in humans. These principles are aligned with the ISAPP consensus definition of probiotics [Hill et al., 2014]. The present study adheres to these standards using a randomized, placebo-controlled human trial complemented by mechanistic *in vitro* and *ex vivo* assays. Although the study focused on healthy adults to minimize confounding variables, future

investigations involving individuals with elevated caries risk or altered glycaemic profiles will enhance the translational relevance of the findings, particularly given the probiotic's dual potential for oral and metabolic health.

7. CONCLUSIONS AND FUTURE PERSPECTIVES

In summary, this study provides robust evidence supporting the multifunctional role of *L. brevis* CD2 in promoting oral health. Through a combination of clinical, biochemical, and microbiological assessments, *L. brevis* CD2 demonstrated significant benefits, including reduced gingival inflammation, modulation of salivary biomarkers, enhanced nitrate-reducing capacity, and inhibition of enzymatic activities linked to cariogenic and metabolic processes. These effects were observed both *in vivo* and *in vitro*, reinforcing the translational potential of this probiotic strain.

The ability of *L. brevis* CD2 to influence key parameters, including salivary flow, pH, buffering capacity, D-lactate levels, sIgA concentration, and α-amylase activity, suggests a broad-spectrum of action that extends beyond oral hygiene and potentially impacts systemic health. Importantly, the nitrate-reducing properties of *L. brevis* CD2 open new avenues for microbiome-based strategies to enhance nitric oxide bioavailability and support cardiovascular and metabolic functions. While the results are encouraging, further studies with larger cohorts, extended follow-up periods, and detailed oral microbiome profiling are needed to confirm long-term efficacy and elucidate the underlying molecular mechanisms. Nonetheless, integrating *L. brevis* CD2 into functional foods or oral care products may represent a practical and safe strategy to enhance oral and systemic health through targeted probiotic therapy.

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