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EFFECTIVENESS OF SUGAR-FREE CHEWING GUM CONTAINING PROBIOTICS ON ORAL HEALTH

MEDS-26/D

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Contents

1.Background 4

2. Study I - Oral Health Benefits of *Heyndrickxia coagulans*: A Systematic Review of Current Evidence 9

2.1 Aim 9

2.2 Materials and Methods 9

2.3 Results 13

2.4 Discussion 33

2.5 Conclusion 35

3. Study II - In vivo study on the salivary kinetics of two probiotic strains delivered via chewing gum 37

3.1 Aim 37

3.2 Materials and Methods 37

3.3 Results 44

3.4 Discussion 49

3.5 Conclusion 52

4. Study III – Assessing the Impact on dental biofilm of *H. coagulans* administered through chewing gum: a double-blind randomized controlled trial 53

4.1 Aim 53

4.2 Material and Methods 53

4.2 Results 59

4.4 Discussion 78

4.5 Conclusions 81

5. Study IV - Chewing Gum added with *H. coagulans* on Halitosis: A Double-Blind, Randomized Controlled Trial 83

5.1 Aim 83

5.2 Material and Methods 83

5.3 Results 85

5.3 Discussion 91

5.4 Conclusion 94

Bibliography 95

1.Background

The human body hosts a complex microbial ecosystem, known as the microbiota, which colonizes various anatomical sites, particularly the gastrointestinal tract and the oral cavity (1,2). The term microbiome, by contrast, refers to the collective genetic material of these microorganisms, encompassing not only their genes but also their interactions with the environment and the human host (3,4). This distinction is critical for understanding the functional and genomic implications of microbial communities.

Under conditions of eubiosis, the microbiota contributes to immunological, metabolic, and inflammatory homeostasis (5). Conversely, dysbiosis, defined as a qualitative and/or quantitative alteration of microbiota, has been associated with a wide range of systemic diseases, including chronic inflammatory disorders, metabolic syndromes, and neuropsychiatric conditions (6).

The development of human microbiota begins prenatally and continues throughout early life (7–9). Among the primary sites of microbial colonization, the oral cavity represents a highly complex and dynamic environment, inhabited by bacteria, fungi, viruses, and archaea (10). Conditions such as dental caries and periodontal disease have been associated with an imbalance in the oral microbiota (11), but the composition of the oral microbiota has been linked not only to oral diseases but also to systemic conditions such as diabetes, obesity, and cardiovascular disease (12,13). Emerging evidence supports a bidirectional relationship between the oral and gut microbiota, with significant implications for the pathogenesis of chronic inflammatory diseases (14,15). In this context, maintaining oral microbial homeostasis is increasingly recognized as a strategic target for promoting systemic health.

Probiotics have recently been proposed as an alternative therapy to promote oral health and counteract the occurrence of gingivitis, halitosis and dental caries.

According to Food and Agriculture Organization (FAO) and World Health Organization (WHO), probiotics were defined as “live microorganisms which, when administered in adequate amounts, confer a health benefit on the host” (16).

According to EFSA and the Italian Ministry of Health, probiotics must fulfil several criteria to be considered safe and effective: they must be non-pathogenic, lack transferable antibiotic resistance genes, be viable at the target site, adhere to and transiently colonize the mucosa, and have beneficial effects demonstrated in well-designed clinical studies (16,17).

Probiotics exert their beneficial effects through multiple mechanisms, including competitive exclusion of pathogens, production of antimicrobial metabolites (organic acids, bacteriocins, hydrogen peroxide), modulation of the host immune system, and enhancement of epithelial barrier function (18). They restore microbial balance after dysbiosis caused by antibiotics, infections, or diet and stimulate mucosal immune responses such as secretory IgA production (18).

Probiotics have demonstrated potential benefits for oral health, including caries, halitosis, and periodontitis, in healthy individuals and those with systemic diseases (19–21). Some of the most studied and promising strains include *Lactobacillus rhamnosus*, *Lactobacillus reuteri, Lactobacillus acidophilus*, *Lactobacillus salivarius, Lactobacillus casei* and *paracasei*, *Bifidobacterium lactis* and various others (22). As evident from the above, all strains have demonstrated beneficial effects on the oral cavity. *Lacticaseibacillus rhamnosus* GG is one of the most extensively investigated, and it has shown its ability to counteract the most prevalent diseases of the gums and teeth, dental caries, and periodontal disease (23–26). In addition, it has shown an inhibitory effect on halitosis-causing bacteria, such as *Porphyromonas gingivalis*, *Tannerella forsythia,* and *Prevotella intermedia* (27) and a strong anticandidal activity (28). All these properties make it a remarkably versatile and effective microorganism for promoting oral health and preventing common oral diseases.

Spore-forming probiotics are experiencing an increase in popularity, attributable to their capacity to enhance survival and stability. In the field of functional food research related to human health, there is an increasing focus on *Bacillus spp*. due to their remarkable tolerance and survivability in the harsh conditions of the gastrointestinal tract. Furthermore, their superior stability during food and pharmaceutical processing and storage renders them ideal candidates for health-promoting formulations (29). In contrast, vegetative probiotic species are more

sensitive to these processes and often require refrigeration to maintain their potency (30).

Heyndrickxia coagulans (formerly *Bacillus coagulans*), have demonstrated antimicrobial, antioxidant, and immunomodulatory properties (31), and it has recently received considerable attention in dentistry (32–34). Recent studies highlight its effectiveness in controlling dental caries by reducing *Streptococcus mutans* and *Lactobacillus spp.* counts in plaque and saliva (30,32). Additionally, they have been shown to lower gingival index scores, reduce bleeding on probing, and combat gingival inflammation (35). *H. coagulans* is listed by the European Food Safety Authority (EFSA) under the Qualified Presumption of Safety status for recommended biological agents. Its use has been approved due to the absence of acquired antimicrobial resistance genes to clinically relevant antibiotics and the lack of toxigenic activity (36), unlike other *Bacillus spp*. (37).

The purpose of this four-phase study is to analyse the release in saliva of probiotics administered through sugar-free chewing gum, test their ability to colonize oral surfaces and investigate their efficacy in reducing halitosis.

The results of the present study will allow the validation of chewing gum as an effective vehicle for the administration of probiotics to improve some important parameters related to oral health. In addition, the ability of the selected probiotic strain to colonize the oral biofilm will be tested. If efficacy is proven, a probiotic-enriched chewing gum may be proposed to help keep mouths healthy. Finally, the results will improve scientific knowledge on the benefits of probiotics for oral health, as there is still no conclusive evidence on this issue.

Microencapsulation is proposed as an effective method for protecting probiotics, enhancing their viability during industrial processing and extending their stability during storage and digestion (38,39).

Probiotics are usually delivered via fermented foods (yogurt, kefir), dietary supplements (capsules, powders, drops), functional foods (fortified juices, cereals), and oral care products (lozenges, sprays) and chewing gum.

Chewing gum has acquired increasing significance in the promotion of oral health over recent decades. Scientific evidence supports four major oral health benefits of

chewing gum: stimulation of saliva production, mechanical cleaning of teeth, pH control, and reduction of cariogenic bacterial load (40,41).

Sugar-free chewing gum positively affects oral health by stimulating saliva flow and promoting natural oral clearance mechanisms (42–45). Moreover, it can serve as a delivery system for ingredients such as xylitol or fluoride, active in reducing dental plaque and the concentration of cariogenic bacteria, such as *mutans Streptococci* and in remineralizing enamel, respectively. (46,47). The EFSA (European Food Safety Authority) has acknowledged the potential benefits of chewing sugar-free gum for oral health, including maintaining tooth mineralization, neutralizing plaque acids, and the reduction of oral dryness. To reap these benefits, the EFSA has recommended that individuals chew 2-3 grams of sugar-free gum for at least 20 minutes, at least three times per day following mealtimes (48–50). Sugar-free chewing gums are composed of a gum base, with the sugar being entirely replaced by alternative bulk sweeteners consisting of one or a combination of several polyols such as sorbitol, mannitol, isomalt, maltitol, maltitol syrup, lactitol, xylitol, and erythritol. High-intensity sweeteners, including acesulfame K, aspartame, cyclamic acid and its sodium and calcium salts, saccharin and its sodium, potassium, and calcium salts, sucralose, thaumatin, neohesperidine dihydrochalcone, and the aspartame-acesulfame salt, are commonly used alone or in combination with other food additives and flavors (51). Moreover, chewing gum is experiencing a surge in popularity as an oral drug delivery system, owing to its ease of use and palatability. Its applications extend to the domains of pain management, smoking cessation, and anti-emetic therapies. (52,53). Chewing gum is particularly suitable for schoolchildren and adolescents, given the high compliance rates observed in these age groups (25).

The administration of probiotics through sugar-free chewing gum has demonstrated encouraging results in enhancing oral health by substantially reducing plaque accumulation, gingival scores, *S. mutans* counts, and bleeding on probing. Furthermore, a reduction in inflammatory mediator levels in gingival crevicular fluid, a major indicator of periodontal disease, has been observed. In addition to these benefits, probiotics have also been found to assist in the alleviation of halitosis (bad breath) (54–58). The benefits mentioned above

suggest that delivering probiotics *via* chewing gum could be an effective adjunct in managing oral conditions.

In this context, this project aims to investigate what is the effect of probiotic strains *H. coagulans* administered through chewing gum on oral heath, through a four-step study:

- Study I: A systematic review to summarize the benefits of *H. coagulans* for oral health;
- Study II: an *in vivo* study to evaluate the salivary kinetics of *H. coagulans* strains delivered *via* chewing gum;
- Study III: a randomized controlled trial to evaluate the ability of *H. coagulans* to colonize and modify dental biofilm;
- Study IV: a randomized controlled trial to evaluate the effect of *H. coagulans* on halitosis.

2. Study I - Oral Health Benefits of *Heyndrickxia coagulans*: A Systematic Review of Current Evidence

2.1 Aim

Heyndrickxia coagulans (formerly *Bacillus coagulans*) is a Gram-positive, facultatively anaerobic bacterium belonging to the *Bacillus* genus, known for its ability to produce lactic acid (59). This microorganism forms heat- and acid-resistant spores, enabling it to survive harsh environmental conditions (60). These features make *H. coagulans* particularly suitable for use as a probiotic in products subjected to industrial processing, including confectionery and functional foods (61).

Given the increasing scientific interest in this probiotic strain, recent studies have begun to explore its potential applications in the prevention and treatment of oral diseases. The aim of this systematic review is to summarize and critically assess the current evidence regarding the effects of *H. coagulans* on oral health.

2.2 Materials and Methods

Protocol and registration

The present systematic review was registered a priori in the International Prospective Register of Systematic Reviews (PROSPERO) under protocol number CRD420251160000 (https://www.crd.york.ac.uk/PROSPERO/view/CRD420251160000) and it has been conducted and reported according to the Cochrane Handbook of Systematic reviews of Interventions and to the guidelines of Preferred Reporting Items for Systematic Review and Meta-Analysis (PRISMA) statement (62,63).

Pico question

To structure the clinical research question and establish the inclusion criteria (64), the PICO model was applied. This review sought to address the following question: 'Does the probiotic *H. coagulans* have an effect on oral health?'

The PICO criteria were defined as follows:

- Population: child and adults.

- Intervention: *H. coagulans* used as probiotic therapy.
- Comparator: placebo or other probiotic or other therapy or any treatment.
- Outcome: all outcomes related to oral health.

Eligibility criteria

Randomized clinical trials (RCTs) and non-randomized studies of interventions (NRSIs) published in English were considered for inclusion. Articles had to be focused on use of *H. coagulans* to improve oral health.

Prospective and retrospective cohort studies, before-and-after comparisons, cross-sectional studies, case reports, case series, non-clinical studies and studies for which full-text articles were not available, were excluded.

Information sources

Two authors performed an electronic literature search from inception to September 1, 2025, using the keywords '*Bacillus coagulans*,' '*Weizmannia coagulans*,' '*Heyndrickxia coagulans*,' and 'probiotics.' Search strings were adapted for each database (Table 2.1). All retrieved records were then imported and consolidated into the screening tool Ryyan® (65).

Table 2.1. Search strings used for electronic literature.

Database	String
Pubmed	("Bacillus coagulans"[MeSH Terms] OR "Bacillus coagulans"[All Fields] OR "Weizmannia coagulans"[All Fields] OR "Heyndrickxia coagulans"[All Fields] OR “coagulans"[Title/Abstract]) AND ("probiotics"[MeSH Terms] OR "probiotic*"[All Fields]) AND (“dent*”[All Fields] OR “gingivitis”[All Fields] OR “periodontitis”[All Fields] OR “dental caries”[All Fields] OR “tooth decay”[All Fields] OR “mutans streptococc*”[All Fields] OR “streptococcus mutans”[All Fields] OR “oral health”[All Fields] OR “oral cavity”[All Fields]
Scopus	TITLE-ABS-KEY ("Bacillus coagulans" OR "Weizmannia coagulans" OR "Heyndrickxia coagulans") AND TITLE-ABS-KEY ("probiotic*") AND TITLE-ABS-KEY(“dent*”OR “gingivitis” OR “periodontitis” OR “dental caries” OR “tooth decay” OR “mutans streptococc*” OR “streptococcus mutans” OR “oral health” OR “oral cavity”)
Embase	(‘Bacillus coagulans’/exp OR ‘Weizmannia coagulans’/exp OR ‘Heyndrickxia coagulans’/exp OR ‘coagulans’/exp) AND (‘probiotic*’/exp) AND (‘dent*’/exp OR ‘gingivitis’/exp

	OR 'periodontitis'/exp OR 'dental caries'/exp OR 'tooth decay'/exp OR 'mutans streptococc*'/exp OR 'streptococcus mutans'/exp OR 'oral health'/exp or 'oral cavity'/exp)

Selection process

Following the removal of duplicate references, study selection was carried out independently and in duplicate by two reviewers. Titles and abstracts were screened, and articles not meeting the eligibility criteria were excluded. Full texts of potentially relevant studies that fulfilled the inclusion criteria were then retrieved and independently assessed by the same two reviewers. Any disagreements or uncertainties were resolved through consultation with a third author.

Data collection process

Data extraction from the included studies was conducted using a customized data collection form (Table 2.2). The following information was recorded: authorship, year and country of publication, journal, study design, sample characteristics (including size, sex, and age), type of intervention and comparator (including dosage of probiotic, mode and timing of administration), and outcomes assessed. Numerical outcome data were extracted and, when possible, rounded to two decimal places; otherwise, data were reported as presented in the original source. Data on bacterial counts expressed in CFU/mL were converted to $\log_{10}$ CFU/mL.

Table 2.2. Customized data collection form used for data extraction from the included studies.

ID	
Author	
Years	
Location	
Journal	
Population	
Gender	
Age means and/or range (year)	
Sample test (number)	
Sample control (n)	
Probiotic intervention	
Delivery	
Amount	
Day of administration	
Placebo/other therapy/control	
Outcomes	
Baseline test (mean)	
Baseline test (SD)	

Follow up test (mean)	
Follow up test (SD)	
Baseline control (mean)	
Baseline control (SD)	
Follow up control (mean)	
Follow up t control (SD)	
p-Value intra-group test	
p-Value intra-group control	
p-Value inter-group	

Risk of bias in individual studies

The risk of bias was independently assessed in duplicate by two reviewers. Any disagreements were resolved by consulting a third reviewer, who provided the final judgment for each study. For randomized controlled trials (RCTs), the revised Cochrane Risk of Bias tool (RoB 2.0) was applied. Responses to signalling questions were entered into the Microsoft Excel® RoB 2 tool, which generated algorithm-based judgments for each domain as low risk, some concerns, or high risk. Visualizations of the results were produced using the Cochrane RoBvis web application (66).

For non-randomized studies the ROBINS-I tool (Risk Of Bias In Non-randomized Studies of Interventions) was used (67).

Summary measures and data synthesis

The sample size together with the number of subjects were extracted for each study. The difference (pre-treatment mean ± SDT0 and post-treatment mean ± SDT1) in the key variables was used to evaluate the effectiveness of the treatment.

For each study, we calculated the mean change from baseline to final assessment for both the treatment and placebo groups. The mean change in the treatment group (Δtest) was defined as the difference between the post-intervention mean and the baseline mean. The same calculation was performed for the placebo group (Δplac). The treatment effect for each study was then expressed as the difference in mean changes between the two groups ($\Delta diff = \Delta test - \Delta plac$).

The standard deviation (SD) of the change scores for each group was estimated using the baseline and final SDs, assuming a correlation coefficient of 0.5 between baseline and final measurements.

The variance of the mean change for each group was obtained by dividing the squared SD of the change by the corresponding sample size. The standard error (SE) of the difference between groups was then calculated as the square root of the sum of these variances.

A meta-analysis was conducted using Stata's meta command, specifying Δdiff as the effect size and SE as its standard error. Forest plots were generated to visually display study-specific treatment effects with their 95% confidence intervals and the pooled overall estimate.

2.3 Results

The outcomes of the database search are illustrated in the flowchart in Figure 2.1. A total of 81 records were initially identified, which decreased to 55 after duplicate removal. Of these, 41 records were excluded by title and abstract screening (Table 2.3), leading to the exclusion of 45 records. As a result, 10 articles were deemed eligible for full-text assessment (Table 2.4). Two records (68,69) corresponded to clinical trial registrations. A search was conducted to verify whether any articles reporting the results of these trials was published. In total, eight studies (70–77) were included in the qualitative synthesis, and three were incorporated into the meta-analysis.

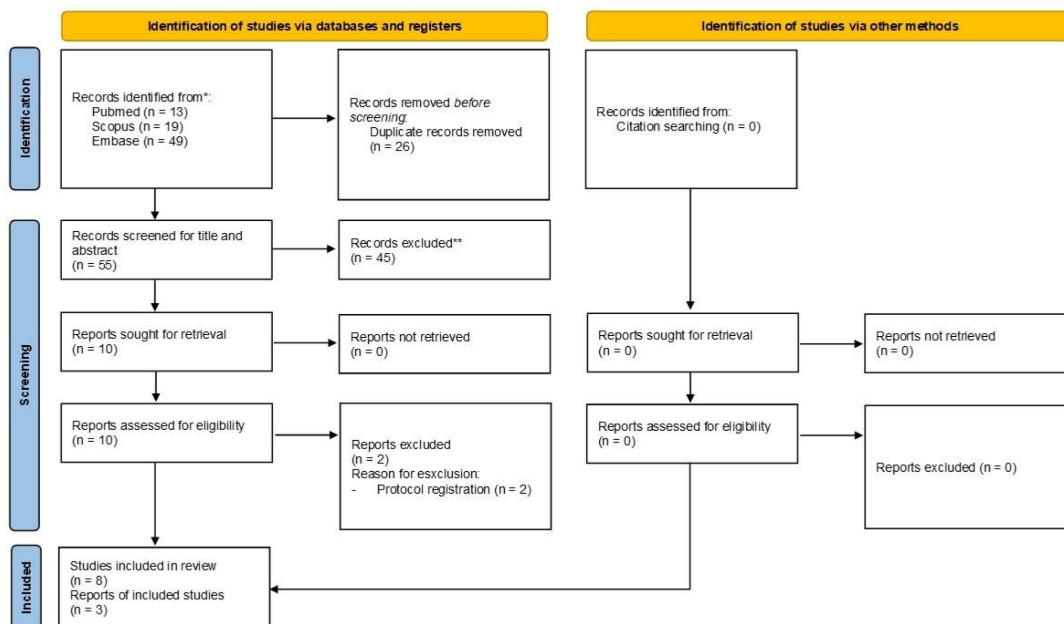

Figure 2.1. Prisma flow chart.

Table 2.3. Title and abstract screening.

ID	Authors	Title	Journal / year	Doi or URL or Protocol registration	Reviewer 1	Reviewer 2
1	A, A.J. and Suresh, A.	Oral microbial shift induced by probiotic Bacillus coagualans along with its clinical perspectives	Journal of Oral Biology and Craniofacial Research / 2023	10.1016/j.jobcr.2023.03.013	Excluded	Excluded
2	Addae, H.Y. and Apprey, C. and Kwarteng, A.	Gut Microbiome-Targeted Nutrition Interventions and Growth among Children in Low- and Middle-Income Countries: A Systematic Review and Meta-Analysis	Curr. Dev. Nutr. / 2024	10.1016/j.cdnut.2024.102085	Excluded	Excluded
3	Banas, J.A. and Popp, E.T.	Recovery of Viable Bacteria from Probiotic Products that Target Oral Health	Probiotics and Antimicrobial Proteins / 2013	10.1007/s12602-013-9142-2	Excluded	Excluded
4	Bhagwat, V.G. and Tattimani, S.V.G. and Baig, M.R.	Dietary Supplementation of Synbiotic Formulation with Phytoactives on Broiler Performance, Relative Ready-to-Cook Weight, Health, Nutrient Digestibility, Gut Health, and Litter Characteristics	J. Appl. Biol. Biotechnol. / 2023	10.7324/JABB.2023.11515	Excluded	Excluded
5	Bogdanović, M. and Mladenović, D. and Mojovic, L. and Djuris, J. and Djukić-Vuković, A.	Intraoral administration of probiotics and postbiotics: An overview of microorganisms and formulation strategies	Brazilian Journal of Pharmaceutical Sciences / 2024	10.1590/s2175-97902024e23272	Excluded	Excluded
6	Bungau, S.G. and Behl, T. and Singh, A. and Sehgal, A. and Singh, S. and Chigurupati, S. and Vijayabalan, S. and Das, S. and Palanimuthu, V.R.	Targeting probiotics in rheumatoid arthritis	Nutrients / 2021	10.3390/nu13103376	Excluded	Excluded
7	Burezq, H.	Feed Additives and their Multiple Beneficial Effects in Sheep Production and Health	Indian Vet. J. / 2022	https://www.researchgate.net/publication/363730948_Feed_Additives_and_their_Multiple_Ben	Excluded	Excluded

				eficial_Effects_in_Sheep_Production_and_Health		
8	Cho, M.-Y. and Eom, J.-H. and Choi, E.-M. and Yang, S.-J. and Lee, D. and Kim, Y.Y. and Kim, H.-S. and Hwang, I.	Recent advances in therapeutic probiotics: insights from human trials	Clinical Microbiology Reviews / 2025	10.1128/cmr.00240-24	Excluded	Excluded
9	Cirio, S. and Salerno, C. and Guglielmetti, S.D. and Mezzasalma, V. and Sarrica, A. and Kirika, N. and Campus, G. and Cagetti, M.G.	In Vivo Study on the Salivary Kinetics of Two Probiotic Strains Delivered via Chewing Gum	Microorganisms / 2025	10.3390/microorganisms13040721	Excluded	Excluded
10	Elghandour, M.M.M.Y. and Pacheco, E.B.F. and Khusro, A. and Tirado-González, D.N. and Lackner, M. and Ponce-Covarrubias, J.L. and De Palo, P. and Maggiolino, A. and Salem, A.Z.M.	Deciphering the role of Moringa oleifera seeds and probiotic bacteria on mitigation of biogas production from ruminants	AMB Express / 2024	10.1186/s13568-024-01744-x	Excluded	Excluded
11	Fu, C. and Shah, A.A. and Khan, R.U. and Khan, M.S. and Wanapat, M.	Emerging trends and applications in health-boosting microorganisms-specific strains for enhancing animal health	Microb. Pathog. / 2023	10.1016/j.micpath.2023.106290	Excluded	Excluded
12	Ghuge, S. and Rahman, Z. and Bhale, N.A. and Dikundwar, A.G. and Dandekar, M.P.	Multistrain probiotic rescinds quinpirole-induced obsessive-compulsive disorder phenotypes by reshaping of microbiota gut-brain axis in rats	Pharmacol. Biochem. Behav. / 2023	10.1016/j.pbb.2023.173652	Excluded	Excluded
13	Izadi, B. and Mohebbi-Fani, M. and Hosseinzadeh,	Alteration of fatty acid profile of milk in Holstein cows fed Bacillus coagulans as probiotic: A	Iran. J. Vet. Res. / 2021	10.22099/IJVR.2021.38159.5558	Excluded	Excluded

	S. and Shekarforoush, S.S. and Nazifi, S. and Rasooli, A.	field study				
14	Jagadeesh, K.M. and Shenoy, N. and Talwar, A. and Shetty, S.	Clinical effect of pro-biotic containing Bacillus coagulans on plaque induced gingivitis: A randomised clinical pilot study	Nitte Univ. J. Health Sci. / 2017	https://www.researchgate.net/publication/340858543_Clinical_effect_of_pro-biotic_containing_Bacillus_coagulans_on_plaque_induced_gingivitis_A_randomised_clinical_pilot_study	Included	Included
15	Ji, M. and Rong, X. and Wu, Y. and Li, H. and Zhao, X. and Zhao, Y. and Guo, X. and Cao, G. and Yang, Y. and Li, B.	Effects of Fermented Liquid Feed with Compound Probiotics on Growth Performance, Meat Quality, and Fecal Microbiota of Growing Pigs	Animals / 2025	10.3390/ani15050733	Excluded	Excluded
16	Jindal, G. and Pandey, R.K. and Agarwal, J. and Singh, M.	A comparative evaluation of probiotics on salivary mutans streptococci counts in Indian children	European archives of paediatric dentistry : official journal of the European Academy of Paediatric Dentistry / 2011	10.1007/BF03262809	Included	Included
17	Joerger, R.D. and Ganguly, A.	Current Status of the Preharvest Application of Proand Prebiotics to Farm Animals to Enhance the Microbial Safety of Animal Products	Microbiol. Spectr. / 2017	10.1128/microbiolspec.PFS-0012-2016	Excluded	Excluded
18	Juárez-Chairez, M.F. and Cid-Gallegos, M.S. and Cristian, C. and Prieto-Contreras, L.F. and Bollain-Y-Goytia, J.J.	The role of microbiota on rheumatoid arthritis onset	International Journal of Rheumatic Diseases / 2024	10.1111/1756-185X.15122	Excluded	Excluded
19	Jurenka, J.S.	Bacillus coagulans	Alternative Medicine	https://pubmed.ncbi.nlm.	Excluded	Excluded

			Review / 2012	nih.gov/225 02625/		
20	Khalesi, S. and Bellissimo, N. and Vandelanotte, C. and Williams, S. and Stanley, D. and Irwin, C.	A review of probiotic supplementation in healthy adults: helpful or hype?	Eur. J. Clin. Nutr. / 2019	10.1038/s41 430-018-0135-9	Excluded	Excluded
21	Koopaie, M. and Fatahzadeh, M. and Jahangir, S. and Bakhtiari, R.	Comparison of the effect of regular and probiotic cake (Bacillus coagulans) on salivary ph and streptococcus mutans count	Dental and Medical Problems / 2019	10.17219/d mp/99757	Included	Included
22	Koopaie, M. and Jahangir, S. and Bakhtiari, R.	Evaluation of the effect of short-term consumption of probiotic (Bacillus coagulans) and ordinary cake on salivary Streptococcus mutans: A pilot study	Journal of Babol University of Medical Sciences / 2018	10.18869/ac adpub.jbum s.20.9.48	Included	Included
23	Krupa, N.C. and Thippeswamy , H.M. and Chandra Shekar, B.R.	Antimicrobial efficacy of Xylitol, Probiotic and Chlorhexidine mouth rinses among children and elderly population at high risk for dental caries - A Randomized Controlled Trial	Journal of Preventive Medicine and Hygiene / 2022	10.15167/24 21-4248/jpmh2 022.63.2.17 72	Included	Included
24	Kuo, C.-L. and Hsin-Hsien Yeh, S. and Chang, T.-M. and I-Chin Wei, A. and Chen, W.-J. and Chu, H.-F. and Tseng, A.-L. and Lin, P.-L. and Lin, Z.-C. and Peng, K.-T. and Liu, J.-F.	Bacillus coagulans BACO-17 ameliorates in vitro and in vivo progression of Rheumatoid arthritis	Int. Immunopha rmacol. / 2024	10.1016/j.in timp.2024.1 12863	Excluded	Excluded
25	Lambo, M.T. and Chang, X. and Liu, D.	The recent trend in the use of multistrain probiotics in livestock production: An overview	Animals / 2021	10.3390/ani 11102805	Excluded	Excluded
26	Lima-Engelmann, K. and Schneider, M.	Probiotic Formulation Development and Local Application with Focus on Local Buccal, Nasal and Pulmonary Application	Curr. Nutraceutic al. / 2022	10.2174/266 5978604666 2211221124 34	Excluded	Excluded
27	Malmir, H. and Ejtahed, H.-S. and	Probiotics as a New Regulator for Bone Health: A Systematic	Evid.-Based Complemen t. Altern.	10.1155/202 1/3582989	Excluded	Excluded

	Soroush, A.-R. and Mortazavian, A.M. and Fahimfar, N. and Ostovar, A. and Esmaillzadeh, A. and Larijani, B. and Hasani-Ranjbar, S.	Review and Meta-Analysis	Med. / 2021			
28	Mansoor, A. and Mansoor, E. and Mehmood, M. and Hassan, S.M.U. and Shah, A.U. and Asjid, U. and Ishtiaq, M. and Jamal, A. and Rai, A. and Palma, P.J.	Novel microbial synthesis of titania nanoparticles using probiotic Bacillus coagulans and its role in enhancing the microhardness of glass ionomer restorative materials	Odontology / the Society of the Nippon Dental University / 2024	10.1007/s10266-024-00921-5	Excluded	Excluded
29	Mazhar, S. and Simon, A. and Khokhlova, E. and Colom, J. and Leeuwendaal, N. and Deaton, J. and Rea, K.	In vitro safety and functional characterization of the novel Bacillus coagulans strain CGI314	Frontiers in Microbiology / 2023	10.3389/fmicb.2023.1302480	Excluded	Excluded
30	McFarlin BK, Deemer SE, Bridgeman EA.	Oral Spore-Based Probiotic Supplementation Alters Post-Prandial Expression of mRNA Associated with Gastrointestinal Health	Biomedicines / 2024	10.3390/biomedicines12102386	Excluded	Excluded
31	McFarlin BK, Henning AL, Bowman EM, Gary MA, Carbajal KM.	Oral spore-based probiotic supplementation was associated with reduced incidence of post-prandial dietary endotoxin, triglycerides, and disease risk biomarkers	World J Gastrointest Pathophysiol. / 2017	10.4291/wjgp.v8.i3.117	Excluded	Excluded
32	McFarlin, B.K. and Henning, A.L. and Bowman, E.M. and Gary, M.M.	Reversing meal-associated gastrointestinal gut permeability issues: Potential treatment target for spore-based probiotics?	Am. J. Gastroenterol. / 2017	10.1038/ajg.2017.309	Excluded	Excluded
33	McFarlin, B.K. and Tanner, E.A. and Hill, D.W. and Vingren, J.L.	Prebiotic/probiotic supplementation resulted in reduced visceral fat and mRNA expression associated with adipose tissue inflammation,	Genes Nutr. / 2022	10.1186/s12263-022-00718-7	Excluded	Excluded

		systemic inflammation, and chronic disease risk				
34	Mitic, K. and Kaftandzieva, A. and Popovska, M. and Ivanovski, K. and Pandilova, M. and Georgieva, S. and Pesevska, S. and Atanasovska-Stojanovska, A. and Kapusevska, B. and Janev, E. and Mijovska, A.	Probiotics and oral health	Res. J. Pharm., Biol. Chem. Sci. / 2017	https://www.embase.com/search/results?subaction=viewrecord&id=L616287824&from=export U2 - L616287824	Included	Included
35	Miyamoto, H. and Seta, M. and Horiuchi, S. and Iwasawa, Y. and Naito, T. and Nishida, A. and Miyamoto, H. and Matsushita, T. and Itoh, K. and Kodama, H.	Potential probiotic thermophiles isolated from mice after compost ingestion	J. Appl. Microbiol. / 2013	10.1111/jam.12131	Excluded	Excluded
36	Mohseni, A.H. and Casolaro, V. and Bermúdez-Humarán, L.G. and Keyvani, H. and Taghinezhad-S, S.	Modulation of the PI3K/Akt/mTOR signaling pathway by probiotics as a fruitful target for orchestrating the immune response	Gut Microbes / 2021	10.1080/19490976.2021.1886844	Excluded	Excluded
37	Mu, Y. and Cong, Y.	Bacillus coagulans and its applications in medicine	Beneficial Microbes / 2019	10.3920/BM2019.0016	Excluded	Excluded
38	NA	Gut Health and the Effect on Substance and Alcohol Cravings	clinicaltrials.gov / 2023	Registration Number NCT06026982	Excluded	Excluded
39	NA	Evaluation of the Effects of Probiotic Toothpastes on Periodontal Health	clinicaltrials.gov / 2024	Registration Number NCT06514664	Included	Included
40	NA	Evaluation of the Consumption of Probiotics on the Bacteria Causing Dental Caries: A Randomised Clinical Trial	clinicaltrials.gov / 2016	Registration Number NCT02752594	Included	Included

41	Nadali, N. and Pahlevanloo, A. and Sarabi, M. and Zomorodi, S.	Production of probiotic powdered barberry (Berberis vulgaris) juice by cast-tape drying technique	LWT / 2023	10.1016/j.lwt.2023.115513	Excluded	Excluded
42	Noman, M. and Kazmi, S.S.U.H. and Saqib, H.S.A. and Fiaz, U. and Pastorino, P. and Barcelò, D. and Tayyab, M. and Liu, W. and Wang, Z. and Yaseen, Z.M.	Harnessing probiotics and prebiotics as eco-friendly solution for cleaner shrimp aquaculture production: A state of the art scientific consensus	Sci. Total Environ. / 2024	10.1016/j.scitotenv.2024.169921	Excluded	Excluded
43	Patel, S. and Patel, M. and Kaushik, G. and Patel, M.S. and Desai, J. and Patel, M.	Innovative Fusion of Probiotics and Mouth Fresheners: Investigating Unaltered Growth, Microscopic Analysis, and Mucoadhesive Strength	Int J Pharm Compd / 2024	https://pubmed.ncbi.nlm.nih.gov/38768505/	Excluded	Excluded
44	Rahmannia, M. and Poudineh, M. and Mirzaei, R. and Aalipour, M.A. and Shahidi Bonjar, A.H. and Goudarzi, M. and Kheradmand, A. and Aslani, H.R. and Sadeghian, M. and Nasiri, M.J. and Sechi, L.A.	Strain-specific effects of probiotics on depression and anxiety: a meta-analysis	Gut Pathogens / 2024	10.1186/s13099-024-00634-8	Excluded	Excluded
45	Ratna Sudha, M. and Neelamraju, J. and Surendra Reddy, M. and Kumar, M.	Evaluation of the Effect of Probiotic Bacillus coagulans Unique IS2 on Mutans Streptococci and Lactobacilli Levels in Saliva and Plaque: A Double-Blind, Randomized, Placebo-Controlled Study in Children	International Journal of Dentistry / 2020	10.1155/2020/8891708	Included	Included
46	Roy, A. and Kumar, Y. and Fatima, S.	A prospective, randomized, single-center, two-arm, open-label study to evaluate the efficacy of biotherapi ®, a two-strain bacillus probiotic blend, as an adjunctive therapy in the	Indian J. Rheumatol. / 2021	10.4103/injr.injr_281_20	Excluded	Excluded

		treatment of rheumatoid arthritis				
47	Rupp, S.K. and Stengel, A.	Bi-Directionality of the Microbiota-Gut-Brain Axis in Patients With Functional Dyspepsia: Relevance of Psychotherapy and Probiotics	Front. Neurosci. / 2022	10.3389/fnins.2022.844564	Excluded	Excluded
48	Siezen, R.J. and Wilson, G.	Probiotics genomics	Microbial Biotechnology / 2010	10.1111/j.1751-7915.2009.00159.x	Excluded	Excluded
49	Sivri, D. and Şeref, B. and Şare Bulut, M. and Gezmen-Karadağ, M.	Evaluation of the Effect of Probiotic Supplementation on Intestinal Barrier Integrity and Epithelial Damage in Colitis Disease: A Systematic Review	Nutrition Reviews / 2025	10.1093/nutrit/nuae180	Excluded	Excluded
50	Spaggiari L, Ardizzoni A, Pedretti N, Iseppi R, Sabia C, Russo R, Kenno S, De Seta F, Pericolini E.	Bacillus coagulans LMG S-24828 Impairs Candida Virulence and Protects Vaginal Epithelial Cells against Candida Infection In Vitro	Microorganisms / 2024	10.3390/microorganisms12081634	Excluded	Excluded
51	Srividya, A.R. and Vishnuvarthan, V.J.	Probiotic: A rational approach to use probiotic as medicine	Int. J. Pharm. Front. Res. / 2011	NA	Excluded	Excluded
52	Verma, A. and Inslicht, S.S. and Bhargava, A.	Gut-Brain Axis: Role of Microbiome, Metabolomics, Hormones, and Stress in Mental Health Disorders	Cells / 2024	10.3390/cells13171436	Excluded	Excluded
53	Wallace, C. and Gordon, M. and Sinopoulou, V. and Akobeng, A.K.	Probiotics for management of functional abdominal pain disorders in children	Cochrane Database Syst. Rev. / 2023	10.1002/14651858.CD012849.pub2	Excluded	Excluded
54	Xie, P. and Luo, M. and Deng, X. and Fan, J. and Xiong, L.	Outcome-Specific Efficacy of Different Probiotic Strains and Mixtures in Irritable Bowel Syndrome: A Systematic Review and Network Meta-Analysis	Nutrients / 2023	10.3390/nu15173856	Excluded	Excluded
55	Yendluru MS, Manne RK, Kannan N, Bepari AS, Anumula A, Pulimi S.	Probiotics an Adjuvant in The Management of Recurrent Aphthous Ulcer: A Randomized Clinical Trial	J Indian Acad Oral Med Radiol / 2020	10.4103/jiaomr.jiaomr_47_20	Included	Included

Table 2.4. Full text screening.

ID	Authors	Title	Journal / year	Doi or URL or Protocol registration	Reviewer 1	Reviewer 2	Reason for exclusion
14	Jagadeesh, K.M. and Shenoy, N. and Talwar, A. and Shetty, S.	Clinical effect of pro-biotic containing Bacillus coagulans on plaque induced gingivitis: A randomised clinical pilot study	Nitte Univ. J. Health Sci. / 2017	https://www.embase.com/search/results?subaction=viewrecord&id=L620487328&from=export U2 - L620487328	Included	Included	
16	Jindal, G. and Pandey, R.K. and Agarwal, J. and Singh, M.	A comparative evaluation of probiotics on salivary mutans streptococci counts in Indian children	European archives of paediatric dentistry : official journal of the European Academy of Paediatric Dentistry / 2011	10.1007/BF03262809	Included	Included	
21	Koopaie, M. and Fatahzadeh, M. and Jahangir, S. and Bakhtiari, R.	Comparison of the effect of regular and probiotic cake (Bacillus coagulans) on salivary ph and streptococcus mutans count	Dental and Medical Problems / 2019	10.17219/dmp/99757	Included	Included	
22	Koopaie, M. and Jahangir, S. and Bakhtiari, R.	Evaluation of the effect of short-term consumption of probiotic (Bacillus coagulans) and ordinary cake on salivary Streptococcus mutans: A pilot study	Journal of Babol University of Medical Sciences / 2018	10.18869/acadpub.jbums.20.9.48	Included	Included	
23	Krupa, N.C. and Thippeswamy, H.M. and Chandra Shekar, B.R.	Antimicrobial efficacy of Xylitol, Probiotic and Chlorhexidine mouth rinses among children and elderly population at high risk for dental caries - A Randomized Controlled Trial	Journal of Preventive Medicine and Hygiene / 2022	10.15167/2421-4248/jpmh2022.63.2.1772	Included	Included	
34	Mitic, K. and Kaftandzieva, A. and Popovska, M.	Probiotics and oral health	Res. J. Pharm., Biol. Chem. Sci. / 2017	https://www.embase.com/search/results?subaction=v	Included	Included	

	and Ivanovski, K. and Pandilova, M. and Georgieva, S. and Pesevska, S. and Atanasovska-Stojanovska, A. and Kapusevska, B. and Janev, E. and Mijovska, A.			iewrecord&id =L61628782 4&from=exp ort U2 - L616287824			
39	NA	Evaluation of the Effects of Probiotic Toothpastes on Periodontal Health	clinicaltrial s.gov / 2024	Registration Number NCT0651466 4	Excluded	Excluded	Registrati on of study protocol
40	NA	Evaluation of the Consumption of Probiotics on the Bacteria Causing Dental Caries: A Randomised Clinical Trial	clinicaltrial s.gov / 2016	Registration Number NCT0275259 4	Excluded	Excluded	Registrati on of study protocol
45	Ratna Sudha, M. and Neelamraju, J. and Surendra Reddy, M. and Kumar, M.	Evaluation of the Effect of Probiotic Bacillus coagulans Unique IS2 on Mutans Streptococci and Lactobacilli Levels in Saliva and Plaque: A Double-Blind, Randomized, Placebo-Controlled Study in Children	Internationa l Journal of Dentistry / 2020	10.1155/202 0/8891708	Included	Included	
50	Yendluru MS, Manne RK, Kannan N, Bepari AS, Anumula A, Pulimi S.	Probiotics an Adjuvant in The Management of Recurrent Aphthous Ulcer: A Randomized Clinical Trial	J Indian Acad Oral Med Radiol / 2020	10.4103/jiao mr.jiaomr_47 _20	Included	Included	

Study types and geographic distribution

The majority of the studies included in the final analysis were conducted in India (70,72,73,76,77) , with two carried out in Iran (74,75) and one in North Macedonia (71). Seven studies were RCT and one was NRSI. All studies included were published between 2017 and 2022. The two registered clinical protocols identified during the search were registered in 2016 (69) and 2024 (68), respectively, and were last updated in 2016 and 2025, respectively. Characteristics of the included studies are shown in Table 2.5.

Samples' sizes and age groups

Findings of the included studies are shown in Table 2.6. Sample sizes of the included studies ranged from 30 (71,75,77) to 183 subjects (74). Two studies included only pediatric patients (72,76); one study included both children and elderly participants (73) and provided separate data for each age group. One study (75) included adolescents and adults, and four studies (70,71,74,77) included only adults. The age of children ranged from 5 to 15 years, while the age of adults ranged from 15 to 73 years.

Table 2.5. Main characteristics of the included studies.

Authors, years	**Journal**	**Country**	**Type of study**	**Funding source**
Jagadeesh et al., 2017 (78)	Nitte University Journal of Health Science	India	RCT	Not reported
Jindal et al., 2011 (76)	European archives of paediatric dentistry	India	RCT	Not reported
Koopaie et al., 2018 (75)	Journal of Babol University of Medical Sciences	Iran	RCT	Not reported
Koopaie et al., 2019 (74)	Dental and Medical Problems	Iran	RCT	International Campus of Tehran University of Medical Sciences (IC-TUMS), Iran
Krupa et al., 2022 (73)	Journal of Preventive Medicine and Hygiene	India	RCT	No funding received
Mitic et al., 2017 (71)	Research Journal of Pharmaceutical, Biological and Chemical Sciences	North Macedonia	NRSI	Not reported
Ratna Sudha et al., 2020 (72)	International Journal of Dentistry	India	RCT	Unique Biotech Limited, Hyderabad, India
Yendluru et al., 2020 (77)	European Journal of Molecular and Clinical Medicine	India	RCT	No funding received

Caries related outcomes

Five studies investigated the ability of H. coagulans to reduce the incidence of dental caries (72–76). In three studies, the population consisted of healthy subjects, and one paper included children (74–76). Two studies enrolled subjects at high risk of caries, one involving a pediatric population (72), and the other including both children and elderly participants (73) (Table 2.6).

In one study (73), H. coagulans was administered in combination with other probiotic strains, whereas in the remaining four studies, it was as a single strain (72,74–76). In three studies the daily dose ranged from 1.50 to 2.00 × 10^9 CFU/mL, with an administration period of 14 days (72,73,76). In two studies, the administration period lasted 7 days; however, the daily probiotic dose was not reported (74,75). In two studies, H. coagulans was administered through the consumption of a cake (74,75); in other two studies (73,76), subjects used a probiotic mouthwash, and finally, in one study (72) the probiotic was delivered via chewable tablets. In three studies the comparator was a placebo (72,74,75); in one study, the probiotic was compared both with a placebo and with another strain (76); while in another study, H. coagulans was compared with chlorhexidine and xylitol (73) (Table 2.6).

Three studies investigated the effect of H. coagulans on salivary S. mutans (MS) levels (74–76), one assessed MS in dental plaque (73), and one evaluated both (72). In healthy adults, salivary Streptococcus mutans counts increased significantly in the placebo group after the intervention period involving cake consumption, whereas no significant change was observed in the group receiving the probiotic (74,75). In the study by Koopaie et al. (74), the difference between the two groups was statically significant at 7-day follow up ($p = 0.03$). Among children, a significant reduction in salivary MS counts was reported in the intervention group but not in the placebo group, both in healthy participants (76) and in those at high caries risk (72). One study compared two different probiotic mouthrinses (H. coagulans vs. L. rhamnosus + Bifidobacterium) and found a significant reduction in salivary MS in both groups (76). At the 14-day follow-up, the reduction in salivary Streptococcus mutans levels was significantly greater in the H. coagulans group compared to the placebo group ($p < 0.001$); however, no significant difference was observed between the two probiotic groups (Table 2.6).

With regard to MS in dental plaque, one study conducted on high caries risk children (72) reported a significant reduction in plaque MS in the probiotic group, but not in the placebo group. In the same study (72) was also evaluated the effect of H. coagulans on Lactobacillus spp. (LB) counts. Results showed a significant reduction of salivary and plaque LB in the probiotic group, whereas a significant increase was observed in the placebo group. In another study (73) H. coagulans was administered as part of a probiotic mouthrinse formulation (L. acidophilus-R0052, L. rhamnosus-R0011, B. longum-R0175, B. coagulans-SNZ1969, S. boulardii) and compared with two alternative treatments: chlorhexidine-based mouthrinse and xylitol-based mouthrinse. All three interventions resulted in a significant reduction of plaque MS, with a greater effect observed in elderly compared to children (Table 2.6).

Finally, two studies (74,75) assessed the effect of H. coagulans on salivary pH, while one study (72) evaluated both salivary and plaque pH. None of the studies reported significant changes in either the probiotic or the placebo group (Table 2.6).

Periodontal related outcomes

One study (77) evaluated the use of H. coagulans in adult patients with plaque-induced gingivitis. The probiotic was administered via mouthwash on 3.00×10^{8} CFU/mL daily dose. After three months of treatment, the Gingival Index (GI), Bleeding on Probing (BOP), and Glutathione Peroxidase (GPx) levels were significantly reduced in the probiotic group ($p< 0.01$), whereas no significant changes were observed in the placebo group. No significant differences in the Plaque Index (PI) were detected in both groups (Table 2.6).

One study (71) investigated the effect of H. coagulans in a group of adult subjects with chronic periodontitis. The probiotic was administered in tablet form in combination with other probiotic strains (H. coagulans, Lactobacillus acidophilus, Streptococcus thermophilus, Lactobacillus bulgaricus, and Bifidobacterium bifidum) at a daily dose of 4.2×10^{9} CFU, following scaling and root planing (SRP). The control group received SRP alone. At two-week follow-up, both groups showed significant improvements in GI, PI, Gingival Bleeding Index (GBI), and Probing Depth (PD). However, intergroup comparisons revealed a statistically significant difference only in GBI, in favor of the probiotic group ($p = 0.04$). Clinical

Attachment Level (CAL) decreased in the control group, whereas it significantly increased in the probiotic group (p = 0.04) (Table 2.6).

Other oral cavity related outcomes

Yendluru et al., 2022 (70) investigated the effect of H. coagulans in combination with tetracyclines on ulcerative lesions in adult patients with Recurrent Aphthous Stomatitis (RAS). The probiotic was administered via mouthwash at the dose of $2.00*10^9$ CFU/mL/day. The control group received tetracyclines alone. After 4 days of treatment, no significant differences were observed in the number or size of lesions between groups; however, the probiotic group reported significantly lower mean pain scores on the Visual Analog Scale (VAS) compared to the control group ($p<0.01$). After 1 week, the probiotic group exhibited a significant reduction in both lesion size ($p<=0.01$) and number ($p=0.02$) compared to the control group, whereas no significant differences in pain scores were noted (Table 2.6).

Table 2.5. Main findings of the included studies.

Author, year	**Population**	**Sample** (size, gender, age range)	**Intervention**	**Outcomes**	**Arm**	**Baseline**	**Follow-up**	**p-Value intragroup**	**p-Value intergroup**
Jindal et al., 2011(76)	Healthy children	N=150 (50/arm)	Mouthwash (*H. coagulans*) 1.50*10^9 CFU/day x 14 days (other probiotic treatment was *L. rhamnosus* + *Bifidobacterium* 1.25*10^9 CFU/day)	Salivary MS ($\log_{10}$ CFU/mL)	Probiotic	Baseline *vs* follow-up: 1.31 x 10^{-7}		<0.001	Probiotic *vs* placebo:<0.001; probiotic *vs* other probiotic:NS
		M/F: 75/75			Other probiotic	Baseline *vs* follow-up: 3.70 x 10^{-5}		<0.001	
		Age range: 7-14 yy			Placebo	na	na	1.00	
Koopaie et al., 2018(74)	Healthy adults	N=30 (15/arm)	Cake (*H. coagulans*) x 7 days	Salivary MS (CFU/mL)	Probiotic	7.87 (1.43) x 10^6	4.65 (0.84) x 10^6	NS	0.032
		M/F: 16/14			Placebo	7.87 (1.43) x 10^6	21.39 (3.90) x 10^6	0.021	
		Age range: 20-68 yy		Salivary pH	Probiotic	7.13 (0.56)	6.91 (0.44)	0.25	na
					Placebo	7.13 (0.56)	6.70 (0.64)	0.06	
Koopaie et al., 2019(74)	Healthy adolescents and adults	N=40 (x-over)	Cake (*H. coagulans*) x 7 days	Salivary MS (CFU/mL)	Probiotic	6.42 (13.53) × 10^6	6.95 (10.42) × 10^6	NS	0.030
		M/F: 21/19			Placebo	6.42 (13.53) × 10^6	1.23 (20.16) × 10^7	0.027	
		Age range: 15-73 yy		Salivary pH	Probiotic	7.13 (0.49)	6.90 (0.23)	NS	na
					Placebo	7.13 (0.49)	7.00 (0.47)	NS	
Krupa et al., 2022(73)	High caries risk children	N=30 (10/arm)	Mouthwash (*L. acidophilus*-R 0052; *L. rhamnosus*-R 0011; *B. longum*-R	Plaque MS ($\log_{10}$ CFU/mL)	Probiotic	6.88 (0.76)	4.97 (2.36)	0.023	na
		M/F: na			Chx	6.43 (1.28)	3.33 (2.71)	0.022	
		Age range: 5-12 yy			Xylitol	6.60 (0.96)	5.67 (1.12)	0.046	

	High caries risk elderly	N=30 (10 per group)	00175; ***B. coagulans***-SNZ 1969; *S. boulardii*) $1.50*10^9$ CFU/day x 14 days		Probiotic	7.16 (0.80)	5.55 (0.43)	0.018	
		M/F: na			Chx	7.13 (1.15)	4.91 (0.73)	0.004	
		Age range: >60 yy			Xylitol	6.42 (1.10)	5.03 (0.47)	0.009	
Ratna Sudha et al., 2020(72)	High caries risk children	48 (24/arm)	Chewable tablet (*H. coagulans*) $2.00*10^9$ CFU/day x 14 days	Plaque LB (log_{10} CFU/mL)	Probiotic	2.71 (0.81)	1.54 (0.72)	<0.001	na
		M/F: 20/28			Placebo	2.72 (0.86)	3.50 (0.35)	<0.05	
		Age range: 5-15 yy		Salivary LB (log_{10} CFU/mL)	Probiotic	3.70 (0.20)	2.94 (0.64)	<0.001	
					Placebo	3.30 (0.47)	3.83 (0.14)	<0.05	
				Plaque MS (log_{10} CFU/mL)	Probiotic	2.33 (0.92)	1.82 (0.78)	<0.05	
					Placebo	2.71 (0.81)	2.56 (0.74)	NS	
				Salivary MS (log_{10} CFU/mL)	Probiotic	3.86 (0.06)	2.56 (0.77)	<0.001	
					Placebo	3.08 (0.61)	3.18 (0.54)	NS	
				Plaque pH	Probiotic	6.00 - 6.30		NS	
					Placebo	6.00 - 6.30		NS	
				Salivary pH	Probiotic	7.60 - 7.80		NS	
					Placebo	7.60 - 7.80		NS	
Yendluru et al., 2020(77)	Adults with RAS	N=31 (17 in intervention/14 in comparison)	Mouthwash (*H. coagulans*) $3.00*10^9$ CFU/day x 7 days	Ulceration size (cm)	Probiotic + TCs	1.24 (0.44)	4 days: 0.35 (0.49)		4 days: 0.110; 7 days: 0.008
		M/F: 15/25					7 days: 0.00 (0.00)		
		Age range: 18-50 yy			TCs	1.36 (0.50)	4 days: 0.64 (0.50)		
							7 days: 0.36 (0.50)		
				N of ulcerazion	Probiotic + TCs	1.12 (0.33)	4 days: 0.53 (0.51)		4 days: 0.580; 7 days: 0.020
							7 days: 0.00 (0.00)		
					TCs	1.21 (0.43)	4 days: 0.43 (0.51)		
							7 days: 0.29 (0.47)		
				Pain (VAS)	Probiotic	2.53 (0.72)	4 days: 0.24 (0.56)		4 days: 0.004; 7

					+ TCs		7 days: 0.00 (0.00)		days: 1.000
					TCs	2.57 (0.65)	4 days: 0.93 (0.73)		
							7 days: 0.00 (0.00)		
Jagadeesh et al., 2017(78)	Adults with plaque induced gingivitis	N=30 (15/arm)	Chewable tablet (*H. coagulans*) $3.00*10^8$ CFU/day x 12 weeks	GI	Probiotic	1.60 (0.80)	1.50 (0.10)	p<0.001	na
		M/F: na			Placebo	1.60 (0.2)	1.60 (0.30)	NS	
		Age range: 18-50 yy		PI	Probiotic	1.50 (0.60)	1.50 (0.20)	NS	
					Placebo	1.40 (0.80);	1.40 (0.70)	NS	
				BOP	Probiotic	81.30 (12.60)	75.89 (11.20)	p<0.0001	
					Placebo	80.50 (20.80)	79.30 (21.20)	NS	
				GPx (pg/ml)	Probiotic	132.90 (21.90)	89.70 (15.50)	p<0.0001	
					Placebo	131.00 (24.90)	131.60 (24.60)	NS	
Mitic et al., 2017 (71)	Adults with chronic periodontitis	N=30 (15/arm)	Tablet (***H. coagulans***, *L. acidophilus, S. thermophilus, L. bulgaricus, B. bifidum*) $4.2*10^9$ CFU/day x 15 days	GI	SRP + probiotic	1.67	0.47	p<0.001	NS
		M/F: na			SRP	1.67	0.47	p<0.001	
		Age range: 18-50 yy		PI	SRP + probiotic	1.73	0.67	p<0.001	NS
					SRP	1.53	0.33	p<0.001	
				GBI	SRP + probiotic	1.33	0.27	p<0.001	NS
					SRP	1.40	0.33	p=0.006	
				PD (mm)	SRP + probiotic	4.93	3.97	p=0.006	0.045
					SRP	5.00	4.73	p=0.003	
				CAL (mm)	SRP + probiotic	4.37	4.20	p=0.044	NS
					SRP	4.20	3.90	p=0.17	

M: male; F: female; SD: standard deviation; N: numbers; MS: *mutans streptococci*; LB: lactobacilli; VAS: Visual Analogue Scale; GI: Gingival Index; PI: Plaque Index; BOP: Bleeding on Probing; GPx: Glutathione Peroxidase; GBI: Gingival Bleeding Index; PD: Probing Depth; CAL: Clinical Attachment Level; Chx: chlorhexidine; TCs: tetracycline; SRP: Scaling and Root Planing; NS: not significant.

Risk of bias assessment

Seven studies (70,72–77) were assessed with the RoB 2.0 tool (Figure 2.2). Five studies (72,74–77) that did not explicitly report the use of an intention-to-treat approach were nonetheless analyzed using the tool for intention-to-treat studies, as they reported no dropouts. Two studies (70,73) were classified as per-protocol. Overall, four studies (72,73,76,77) showed low risk of bias, two (74,75) raised some concerns, and one (70) had high risk of bias, mainly related to the selection of reported results due to the absence of protocol pre-registration and clear specification of planned analyses. One additional study (71), evaluated with ROBINS-I, was judged to raise some concerns (Figure 2.3).

Intention-to-treat	Authors, year	Experimental	Compara	Outcome	D1a	D1b	D2	D3	D4	D5	Overall
	Jagadeesh et al., 2017	*H. coagulans*	Placebo	Gingival index	+	+	+	+	+	+	+
	Jindal et al., 2011	*H. coagulans*	Placebo	Salivary MS	+	+	+	+	+	+	+
	Koopaie et al., 2018	*H. coagulans*	Placebo	Salivary MS	!	+	+	+	+	!	!
	Koopaie et al., 2019	*H. coagulans*	Placebo	Salivary MS	+	+	+	+	+	!	!
	Ratna Sudha et al., 20	*H. coagulans*	Placebo	Salivary MS	+	+	+	+	+	+	+

+ Low risk
! Some concerns
High risk

D1a Randomisation process
D1b Timing of identification or recruitment of participants
D2 Deviations from the intended interventions
D3 Missing outcome data
D4 Measurement of the outcome
D5 Selection of the reported result

Per-protocol	Authors, year	Experimental	Comparator	Outcome	D1a	D1b	D2	D3	D4	D5	Overall
		L. acidophilus-R 0052; L. rhamnosus-R 0011;			+	+	+	+	+	+	+

Figure 2.2. Risk of bias assessment of RCT (RoB 2.0).

Authors, year	Experimental	Comparator	Outcome
Mitic et al. 2017	Scaling and Root Planing *(SRP)* + *H. coagulans*, *L. acidophilus*, *S. thermophilus*, *L. bulgaricus*, *B. bifidum*	Scaling and Root Planing (SRP)	Probing Depth (PD)

Domain	Judgment	Comment
Bias due to confounding	Moderate	The study excluded smokers and patients with systemic diseases, but did not use multivariate analyses to control for other confounders.
Bias in selection of participants into the study	Low	Clear and enforced inclusion/exclusion criteria prior to surgery.
Bias in classification of interventions	Low	Well-defined intervention with standardized composition, dosage and duration.
Bias due to deviations from intended interventions	Moderate	No compliance monitoring and no blinding.
Bias due to missing data	Low	No drop-outs were reported and all patients completed the study.
Bias in measurement of outcomes	Moderate	Standardized clinical outcomes, but it is unclear whether the assessors were treatment-blind.
Bias in selection of the reported result	Moderate	Key results reported, but no pre-registered protocols mentioned.
Overall	**Moderate**	

Figure 2.3. Risk of bias assessment of NRSI (ROBINS-I).

Meta-analysis

Three studies were included in the meta-analysis (Figure 2.4), which represented the only quantitative synthesis feasible based on the available data. The outcome investigated was the reduction of salivary S. mutans. The outcome investigated was the reduction of salivary S. mutans. Individual study effect sizes ranged from −0.25 (95% CI: −0.41 to −0.09) to −1.40 (95% CI: −1.78 to −1.02), all favoring the probiotic intervention over control.

The pooled analysis demonstrated an effect size of −0.74 (95% CI: −1.38 to −0.10; $p = 0.02$), suggesting that the intervention had a statistically significant beneficial effect. However, heterogeneity was substantial ($\tau^2 = 0.31$; $I^2 = 98.27\%$; $H^2 = 57.74$; $Q(2) = 39.76$, $p < 0.001$), indicating considerable variability among the included studies. Despite this high heterogeneity, the

direction of effect was consistent across all included studies, supporting a potential beneficial role of H. coagulans in reducing salivary S. mutans levels.

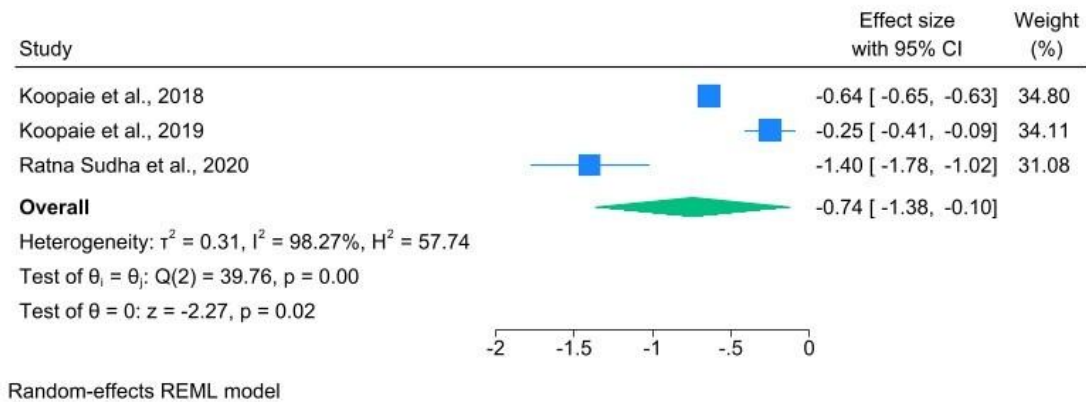

Figure 2.4. Forest plot for salivary *mutans* Streptococci in children and adults.

2.4 Discussion

This systematic review synthesized the available literature on the effects of Heyndrickxia coagulans (commonly previously referred to as Bacillus coagulans) on oral health outcomes. Across six randomized controlled trials, H. coagulans demonstrated promising benefits, most notably in reducing salivary Streptococcus mutans levels and improving selected periodontal parameters. These findings suggest that H. coagulans may have a potential role as an adjunctive strategy for caries prevention and the management of gingival inflammation. However, these conclusions should be interpreted with caution due to the limited number of studies available, as well as their heterogeneity and methodological limitations.

The most consistent finding across the included trials was a significant reduction in Streptococcus mutans counts following H. coagulans administration, particularly among children and individuals at high risk of caries. The pooled analysis of four studies demonstrated a significant effect size, despite substantial heterogeneity. These results support the hypothesis that H. coagulans can meaningfully modulate the salivary microbiota. The effects on Lactobacillus spp. were less conclusive, with only one study reporting significant reductions. Considering that Lactobacilli spp. is primarily involved in the progression of dentinal caries rather than the initiation of the caries

process, this partial effect may still hold clinical relevance, though further confirmation in larger, well-designed trials is warranted.

The impact of H. coagulans on plaque accumulation and gingival inflammation was variable. Some studies reported stabilization or reduction of plaque indices compared with control groups, while others found no difference. Notably, in gingivitis and periodontitis, H. coagulans was associated with improvements in gingival index, bleeding on probing, and clinical attachment level. These results are encouraging and suggest a possible role in modulating host inflammatory response.

The results of this review are consistent with broader probiotic research, which has repeatedly shown that probiotics can reduce S. mutans levels in saliva and plaque (79,80). However, evidence for effects on Lactobacilli and on clinical indices such as plaque and gingival scores has been mixed (81,82). Several meta-analyses have reported modest reductions in gingival inflammation with probiotics, but heterogeneity remains high, and the evidence is often rated as low (83,84). By focusing specifically on H. coagulans, the present review adds clarity about the potential of this species, which may have unique properties, including spore formation that enhances survival and colonization.

Several mechanisms may explain the observed effects. H. coagulans may inhibit cariogenic bacteria through competitive exclusion, production of antimicrobial peptides, and disruption of biofilm formation (85). In addition, it may exert anti-inflammatory effects by modulating cytokine production and oxidative stress markers, as evidenced by reductions in gingival bleeding and Glutathione Peroxidase activity in some trials (78). Unlike chemical antiseptics such as chlorhexidine, probiotics aim to restore microbial balance rather than indiscriminately suppress the oral microbiota, which could explain their favorable safety profile.

The evidence base is strengthened by the predominance of randomized controlled trials, the inclusion of both pediatric and adult populations, and the use of multiple delivery vehicles (tablets, mouthwash, food matrices). Nonetheless, several limitations must be acknowledged. The studies were highly

heterogeneous in strain dosage, intervention duration, outcome measures, and control conditions, which precluded robust meta-analysis for most outcomes. Sample sizes were generally limited, and several studies exhibited an unclear or high risk of bias, primarily due to insufficient reporting of randomization procedures and the absence of blinding, which may have introduced potential methodological weaknesses. Moreover, follow-up periods were quite short, reducing insights into the durability of probiotic effects.

Despite these limitations, the findings may have relevant clinical implications. H. coagulans could serve as a safe adjunct to conventional caries prevention measures, particularly in children at high risk for caries development. Its use in mouthwash or tablets may offer practical advantages for daily application and better compliance. The observed improvements in gingival inflammation indicate potential benefits for individuals with mild periodontal disease, positioning H. coagulans as a useful adjunct to conventional mechanical plaque control and professional prophylaxis. Notably, no significant adverse effects were reported, further supporting its safety and potential suitability for long-term preventive applications.

Further well-designed randomized controlled trials are necessary to confirm these preliminary results and to address remaining gaps. Future studies should standardize probiotic dosages, use validated outcome measures, and include longer follow-up periods to assess persistence of effects. Investigations employing next-generation sequencing could provide deeper insight into changes in the oral microbiome and clarify whether H. coagulans promotes a sustainable shift toward health-associated microbial communities. Trials evaluating caries incidence, periodontal disease progression, and patient-reported outcomes would help determine the true clinical significance of the observed microbiological changes.

2.5 Conclusion

Overall, this systematic review highlights that, to date, there is no conclusive evidence supporting the clinical efficacy of H. coagulans in improving oral health. However, the currently available data in the literature provide encouraging indications that justify optimism for its potential future role in oral

care. Moreover, rigorous, large-scale clinical studies are warranted to establish optimal dosages, delivery systems, and long-term efficacy, ultimately defining the place of H. coagulans in preventive and therapeutic oral health care.

3. Study II - In vivo study on the salivary kinetics of two probiotic strains delivered via chewing gum

3.1 Aim

As proposed in several studies (55,57,58,86,87), the benefits on delivering probiotics via chewing gum could be an effective adjunct in managing oral conditions. Although studies have demonstrated the clinical efficacy of sugar-free probiotic chewing gum, it is imperative to ascertain the amount of the administered probiotic that remains in the oral cavity, as this directly influences its ability to colonize oral surfaces and ensure a lasting effect (88,89). While the release and concentration of various active agents delivered via chewing gum have been investigated in both in vitro and in vivo studies (47,90), no research has evaluated the release kinetics of probiotics from chewing gum to date. Therefore, an in vivo microbiological study was designed to analyse the kinetics of probiotics in saliva in 2-hours follow-up, following their administration through sugar-free chewing gum. The hypothesis to be tested is that the probiotic administered through chewing gum remains in saliva long enough for it to adhere to oral surfaces (91).

3.2 Materials and Methods

Design of the study

This randomized, cross-over microbiological study was designed and conducted at the Department of Biomedical, Surgical and Dental Sciences, University of Milan, Milan, Italy, following the principles of the Declaration of Helsinki. The Ethical Committee of the University of Milan approved the study (13/02/2024, no. 24/24). Recruitment of participants, Intervention, and microbiological measurements were carried out between March and May 2024.

Sample selection

The study was conducted on healthy adult volunteers selected from the staff of the Perfetti Van Melle SpA (PVM, Lainate, Italy).

No prior studies with the same objective were identified, so a sample size calculation could not be performed. Therefore, conducting an in vivo microbiological study with 10 participants was decided. The inclusion criteria were adult subjects aged 18 to 64 years, at least 24 natural teeth (excluding third molars), gingival index and plaque index scores ≤ 2, and a stimulated salivary flow rate between 1.5 and 2.0 mL/min. Exclusion criteria included the presence of systemic diseases, pregnancy or lactation, history of drug abuse, smoking habits, use of fixed orthodontic appliances, and allergies to any ingredients in the chewing gums used. An email explaining the purpose of the study and inviting participation was sent to all staff at the PVM site in Lainate. Thirteen individuals who consented to participate were interviewed to assess their eligibility based on the inclusion and exclusion criteria. They were then examined by a calibrated dentist (SC) to obtain their gingival index scores (92) gingival index (93) and stimulated salivary flow rate. Ten eligible subjects were identified and enrolled. All study participants gave their written consent to participate.

Chewing gums production

All chewing gums used in the study were produced and supplied by PMV (Figure 3.1).

The sugar-free chewing gums (weight 2.1g) were formulated with gum base (Gum Base Co.), food-grade polyols, excluding xylitol (proprietary blend; manufactured by Roquette Frères S.A. and Cargill Srl), food-grade intensive sweeteners (Ajinomoto Co., Inc,), flavors (Mondarom Selegroven AG) and incorporated specific probiotic strains under investigation. These included *Lacticaseibacillus rhamnosus* GG provided either in its non-microencapsulated form (LGG®, DSMZ code: DSM 33156, supplied by Chr. Hansen, Boege Alle 10-12, 2970 Hoersholm, Denmark) or as microencapsulated cell (Encaptimus™, ATCC 53103, provided by AnaBio Technologies LTD, 11 Herbert Street, Dublin, D02 RW27, Ireland. Containing: Maltodextrin, *Lactobacillus rhamnosus*, Coconut Oil, Pea Protein Isolate, Polysorbate 20) [55], and *Heyndrickxia coagulans* SNZ1969 (*Heyndrickxia coagulans* SNZ1969®, provided by Sanzyme Biologics Ltd (Sattva Signature Tower, H.No. 8-2-

472/1/A/B/SF-3, Road No. 1, Banjara Hills, Hyderabad, 500034 Telangana, India), which was added in its spore form. Therefore, three different chewing gums were produced.

Figure 31. Chewing gum used in the study.

The production process of the chewing gum is summarized in Figure 3.2. Initially, the gum base is melted at 50°C and combined with polyols, artificial intense sweeteners, and flavourings sequentially, achieving a homogeneous mixture. The freeze-dried probiotic biomass is incorporated as a final component below 50° C to preserve its activity. After mixing, in the rolling and scoring system, a mass of gum is extruded into a thick slab, which is then worked into a thinner and foil by a series of rollers. Finally, the foil is shaped into single pieces by one or more cutting rollers. These pieces undergo cooling in a conditioning room before being panned, and afterward, individual pieces are prepared for packaging (94).

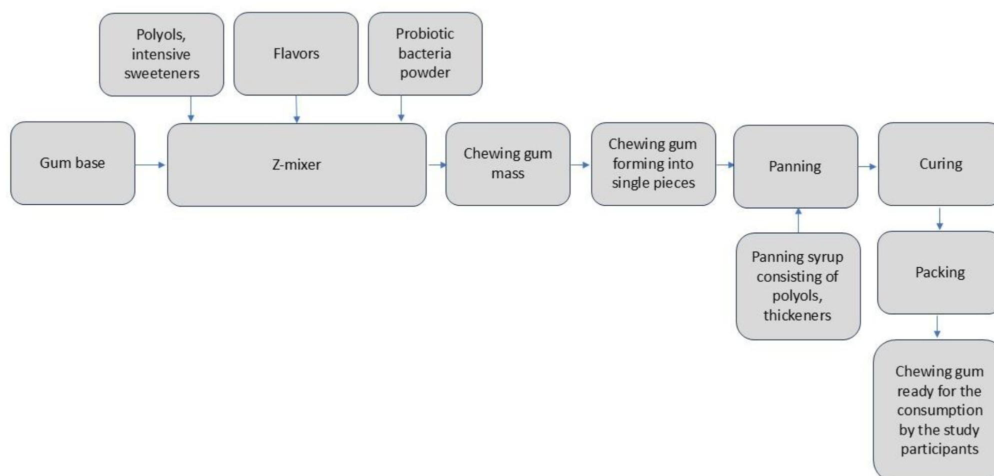

Figure 3.2. The chewing gum production process.

At the end of the production process, the amount of Colony Forming Units (CFUs) contained in the chewing gum was assessed for each probiotic strain according to the following protocol. The gum pieces in the bag were manually crushed and then processed in a Stomacher® (Stomacher® 3500 peristaltic homogenizer, Seward, West Sussex, United Kingdom) for 2 min. The resulting homogenized chewing gum mass was further serially diluted 1:10 in Maximum Recovery Diluent (MRD) buffer (GranuCult® prime Peptone salt solution - Maximum recovery diluent, Merck KGaA, Darmstadt, Germany). Aliquots of 100 μL were plated onto selective media: GYEA-agar (Glycerol 5 g/L, Yeast extract 2 g/L, K2HPO4 1 g/L, BromoCresol Green 10 ml/l from a stock of 5 g/L, Agar 15 g/L pH 5.5, Merck KGaA, Darmstadt, Germany) for *H. coagulans* SNZ1969® and RVB-MRS-agar (*Lactobacilli* MRS w/o Dextrose 3515 g/L, Condalab, Madrid, Spain; Rhamnose 20 g/L, Bromocresol green 10 ml/l from a stock of 5 g/L, Agar 15 g/L pH 5.5 after autoclave, Cysteine-HCl 0.05%, Vancomycin 50 mg/L, Merck KGaA, Darmstadt, Germany) for *L. rhamnosus* GG). For *H. coagulans* SNZ1969®, part of the samples underwent viable count after pasteurization (incubation in a water bath at 90° C for 10 min) to quantify bacterial spores. The plates were incubated at 37° C for 72 h for the evaluation of *L. rhamnosus* GG and at 55° C for 72 h in anaerobic conditions, established by incubating the plates in AnaeroJar Oxoid 2.5 L jars (Thermo Fisher Scientific™, Waltham, Massachusetts, USA) containing Anaerocult™ A (Merck KGaA, Darmstadt, Germany), for the assessment of *H. coagulans* SNZ1969® CFUs. The procedures described were repeated for each sample in triplicate. CFUs were identified by morphology and color and finally counted. The viable bacterial cell counts were expressed as colony-forming units /gram (CFU/g).

At the end of the production process, the mean counts of probiotics in one pellet of chewing gum were:

- 6×10^8 CFU of *Lacticaseibacillus rhamnosus* LGG® (non-microencapsulated form);
- 2×10^8 CFU of *Lacticaseibacillus rhamnosus* GG (microencapsulated);
- 5×10^8 CFU of *Heyndrickxia coagulans* SNZ1969®.

Use of chewing gum

Participants were instructed to chew a pellet of gum containing one of the probiotic strains. Following a washout period of one week, they were asked to chew a second pellet containing a different strain. After another one-week washout period, they chewed the third and final pellet. At each visit, each subject was administered only one formulation per session in a randomized crossover design to ensure that the efficacy of each formulation was assessed independently. Each subject drew one of three chewing gums at the first appointment using a lottery system. They drew one of the two remaining gums at the second appointment, and the last remaining gum was administered at the third appointment. Administration occurred in the morning, at least two hours after breakfast and oral hygiene routines.

Both participants and investigators were blinded to the specific probiotic strain in each gum. Volunteers were instructed to chew the gum for 10 min and to refrain from eating or drinking anything for the subsequent two hours.

Saliva samples of at least 0.5 mL were collected from the floor of the mouth using sterile disposables at the following time points: before chewing gum use (T_0), and at 1, 5, 10, and 20 min, as well as 1 and 2 h after the procedure began (T_1–T_6). Samples were stored at 4° C, transported to the laboratory, and processed within two hours.

An investigator (SC) conducted brief telephone interviews with the participants on the evening of the intervention day and one week later to record any side effects associated with the chewing gum administered.

The flow chart of the study design is presented in Figure 3.3.

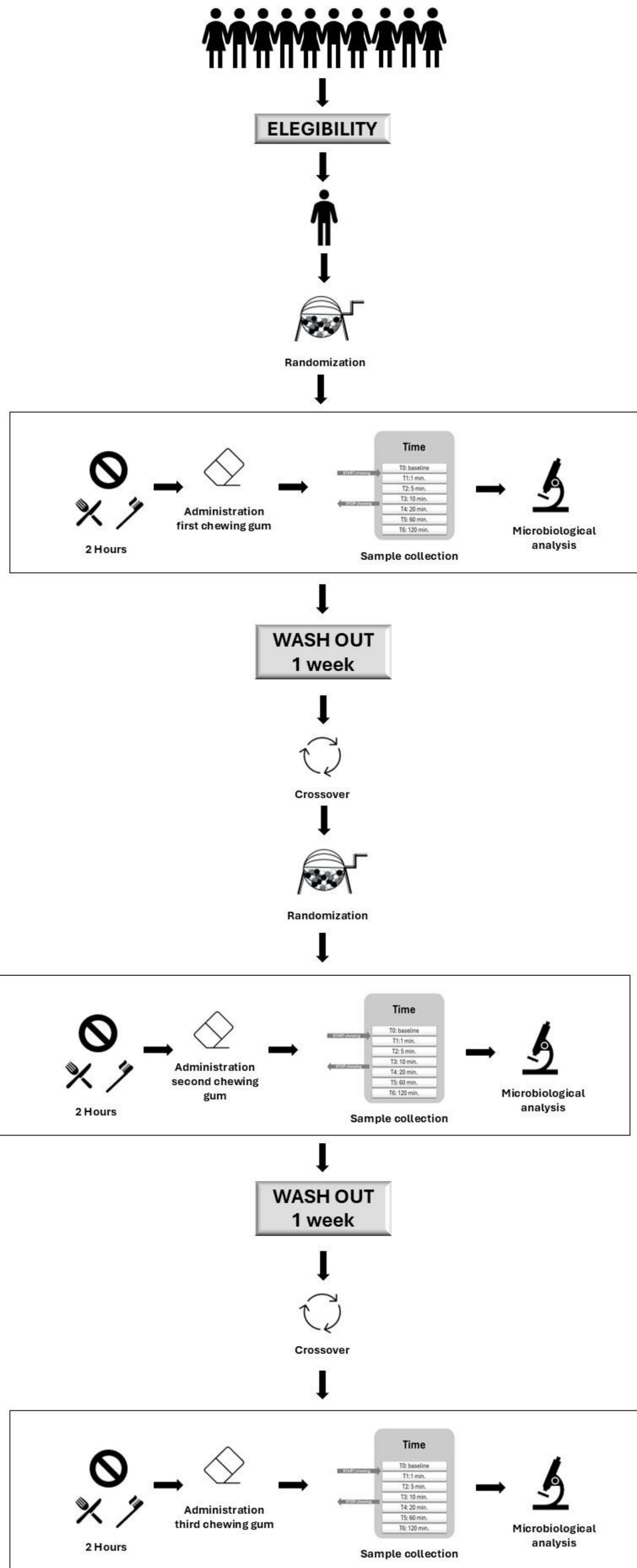

Figure 3.3. Flow chart of the study design.

At the first appointment, each participant chewed one randomly selected chewing gum from the three gums tested in the study. At the second appointment, they chewed one of the two remaining gums, and at the final appointment, the last remaining gum. This process was repeated for each participant, ensuring that all three gums were tested in a randomized order across the different subjects. Each chewing gum was chewed two hours after breakfast and the oral hygiene routine. Salivary samples were then collected over two hours and analysed microbiologically.

Mycrobiologically analysis

Aliquots of 300 µL of saliva were diluted in 600 µL of MDR buffer (GranuCult® prime Peptone salt solution - Maximum recovery diluent, Merck KGaA, Darmstadt, Germany). Samples were processed as it was explained for chewing gum analysis, but without any preventive treatment to break down the microcapsules. If different morphologies were detected, three colonies per type were selected and analysed by colony polymerase chain reaction (PCR), picking the colony into a PCR reaction with the strain-specific primers PVM-Wc-1F 5'-TTGTCTTTGGATCAGTTACAG-3' and PVM-Wc-1R 5'-GCATAGGAATACCTTGTGCA-3' for *H. coagulans* SNZ1969® (95) and the primers GG I 5'-CAATCTGAATGAACAGTTGTC-3' and GG II 5'-TATCTTGACCAAACTTGACG-3' for *L. rhamnosus* GG (96). Morphologies that revealed expected amplicons by agarose gel electrophoresis were confirmed as CFU and included in the final count. Some of the amplicons obtained were confirmed by Sanger sequencing (97). The amount of viable *H. coagulans* SNZ1969® (without spores) was obtained by subtracting the total *H. coagulans* colony count minus the colonies of pasteurized *H. coagulans* SNZ1969® (spores) for each interval.

Statistical analysis

All data were transformed into a logarithmic scale to normalize the distribution. Values between 0 and 1 were rounded to 1 before the log transformation.

The ANOVA test was applied. When the variance, evaluated with Bartlet's equal-variances test, was not equal, Welch's t test was used to assess differences

between probiotics. The Tukey's range test was applied to make pairwise comparisons between different probiotics at every time point, different time points of the same probiotic, and between the area under the curve of different probiotics. The area under the curve was calculated to quantify the overall exposure of the subjects' oral cavity to probiotic strains over time using the following STATA commands: mean_log_counts of the different probiotics and then the mean trapezoid_area, over the probiotic counts. Cuzick's test with rank scores was used to describe trends within each strain during time. All data were analysed using STATA® software (v18 for Mac). Statistical significance was set at α = 0.05 for all analyses.

3.3 Results

All included subjects, 6 females and 4 males, age range 23–52 years (mean age 36.4 ±10.0), completed the study. No adverse effects were reported by the participants or noted by the investigators during the Intervention.

At T_0, none of the probiotics tested was detected in saliva. At T_1, the highest counts value was registered for all probiotic strains, followed by a constant decrease until T_6.

L. rhamnosus GG in microencapsulated form showed the lowest salivary counts at each time point (p<0.01) (Table 3.1; Figure 3.4).

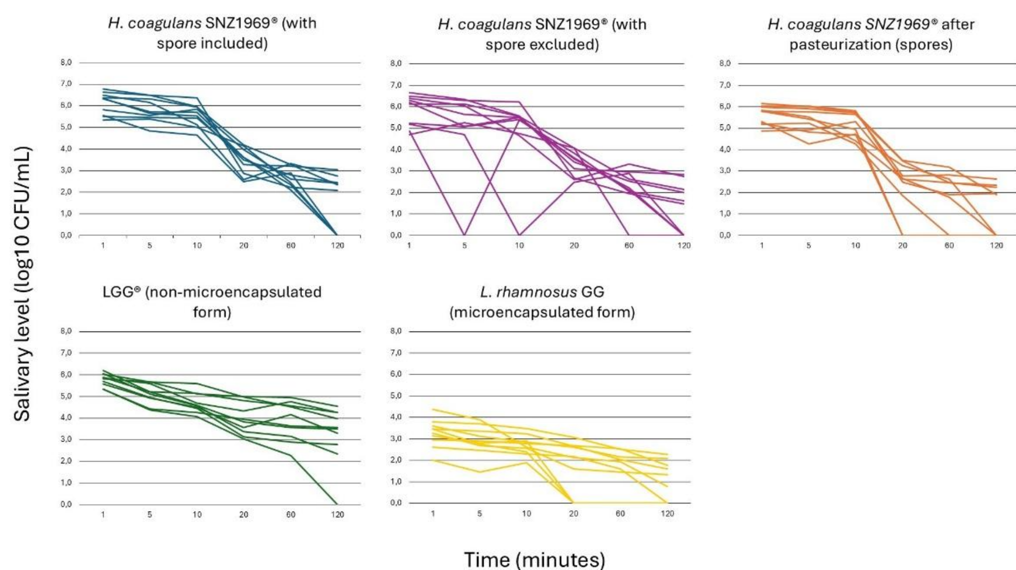

Figure 3.4. Viable count kinetics of probiotic cells in salivary samples.

Table 3.1. Salivary counts (log10 CFU/mL) at different time points of the probiotic strains.

Time	Probiotic strain	***H. coagulans* SNZ1969® with spores included** (log$_{10}$ CFU/mL)	***H. coagulans* SNZ1969® pasteurized** (log$_{10}$ CFU/mL)	***H. coagulans* SNZ1969® with spores excluded** (log$_{10}$ CFU/mL)	**LGG® (free form)** (log$_{10}$ CFU/mL)	***L. rhamnosus* GG in micro-encapsulated form** (log$_{10}$ CFU/mL)	**p-value**	
T_0	Mean±SD	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0		
	Median	0.0	0.0	0.0	0.0	0.0		
	Min-Max	0.0; 0.0	0.0; 0.0	0.0; 0.0	0.0; 0.0	0.0; 0.0		
	95% CI	0.0; 0.0	0.0; 0.0	0.0; 0.0	0.0; 0.0	0.0; 0.0		
T_1	Mean±SD	6.1±0.5	5.6±0.4	5.8±0.7	5.8±0.3	3.3±0.6	**<0.01[a*]**	**<0.01[d µ]**
	Median	6.3	5.8	6.2	5.8	3.4	0.74[b µ]	0.64[e µ]
	Min-Max	5.4; 6.8	4.9; 6.2	4.7; 6.7	5.3; 6.2	2.0; 4.4	0.92[c µ]	
	95% CI	5.8; 6.5	5.3; 5.9	5.3; 6.4	5.6; 6.0	2.8; 3.7		
T_2	Mean±SD	5.8±0.5	5.4±0.1	5.1±1.8	5.1±0.4	2.9±0.7	**<0.01[a§]**	**<0.01[d µ]**
	Median	5.7	5.5	5.4	5.2	2.9	0.43[b µ]	0.52[e µ]
	Min-Max	4.9; 6.5	4.3; 6.0	0.0; 6.3	4.4; 5.7	1.5; 3.9	0.94[c µ]	
	95% CI	5.4; 6.2	5.0; 5.8	3.7; 6.4	4.8; 5.4	2.4; 3.4		
T_3	Mean±SD	5.6±0.5	5.1±0.6	4.9±1.7	4.7±0.4	2.7±0.4	**<0.01[a§]**	**<0.01[d µ]**
	Median	5.6	5.1	5.5	4.6	2.7	0.43[b µ]	0.24[e µ]
	Min-Max	4.6; 6.4	4.3; 5.8	0.0; 6.2	4.1; 5.6	1.9; 3.5	0.96[c µ]	
	95% CI	5.2; 5.9	4.7; 5.5	3.6; 6.1	4.4; 5.0	2.4; 3.0		
T_4	Mean±SD	3.4±0.6	2.3±1.2	3.3±0.6	4.0±0.7	1.7±1.2	**<0.01[a§]**	**<0.01[d µ]**
	Median	3.6	2.6	3.5	3.9	2.2	1.00[b µ]	0.67[e µ]
	Min-Max	2.5; 4.2	0.0; 3.5	2.5; 4.1	3.0; 5.0	0.0; 3.1	**0.01[c µ]**	
	95% CI	3.0; 3.9	1.3; 3.2	2.9; 3.8	3.5; 4.5	0.8; 2.6		
T_5	Mean±SD	2.7±0.4	1.7±1.2	2.3±0.9	3.8±0.9	1.4±1.0	**<0.01[a*]**	**<0.01[d µ]**
	Median	2.7	2.2	2.4	3.9	1.8	0.82[b µ]	0.08[e µ]
	Min-Max	2.1; 3.3	0.0; 3.2	0.0; 3.3	2.3; 4.9	0.0; 2.5	0.71[c µ]	
	95% CI	2.4; 3.0	0.8; 2.6	1.6; 2.9	3.2; 4.5	0.7; 2.2		
T_6	Mean±SD	1.5±1.3	1.11±1.12	1.3±1.1	3.3±1.3	1.0±1.0	**<0.01[a*]**	**<0.01[d µ]**
	Median	2.2	1.0	1.5	3.5	1.1	0.99[b µ]	**0.02[e µ]**
	Min-Max	0.0; 3.1	0.0; 2.6	0.0; 2.9	0.0; 4.5	0.0; 2.3	1.00[c µ]	
	95% CI	0.6; 2.5	0.3; 2.0	0.4; 2.1	2.3; 4.2	0.3; 1.7		
p-value	T_{all}	**<0.01§**	**<0.01§**	**<0.01§**	**<0.01***	**<0.01§**		
	T_1 *vs* T_2 µ	0.93	1.00	0.42	0.95	0.76		
	T_2 *vs* T_3 µ	0.97	0.99	0.84	0.99	1.00		
	T_3 *vs* T_4 µ	**<0.01**	**<0.01**	0.35	0.12	0.10		

	T_4 *vs* T_5 $^{\mu}$	0.27	0.81	1.00	0.98	0.42
	T_5 *vs* T_6 $^{\mu}$	**<0.01**	0.70	0.53	0.87	0.52
Trend$^{¥}$	z	-6.20	-5.69	-4.85	-5.02	-5.64
	p	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**

* Anova; § Welch's t-test; µ Tukey's range test; ¥ Cuzick's test with rank scores; a LGG® (free form) vs L. rhamnosus GG in microcapsulated vs H. coagulans SNZ1969® with spores included vs H. coagulans SNZ1969® with spores excluded vs H. coagulans SNZ1969® pasteurized; b H. coagulans SNZ1969® with spores included vs H. coagulans SNZ1969® with spores excluded; c H. coagulans SNZ1969® with spores excluded vs H. coagulans SNZ1969® pasteurized; d LGG® (free form) vs L. rhamnosus GG in microencapsulated form; e LGG® (free form) vs H. coagulans SNZ1969® with spores included; Tall : T1 vs T2 vs T3 vs T4 vs T5 vs T6; SD: Standard Deviation; CI: Confidence Interval.

When comparing *H. coagulans* SNZ1969® and *L. rhamnosus* LGG® in non-microencapsulated form, the total viable counts of *H. coagulans* SNZ1969® (i.e., spores + vegetative cells) were higher at T_1, T_2, and T_3. In comparison, the viable counts of *L. rhamnosus* LGG® were higher at T_4, T_5, and T_6. However, no significant differences were observed between the two probiotic strains (p=0.64 at T_1; p=0.52 at T_2; p=0.24 at T_3; p=0.67 at T_4; p=0.08 at T_5; p=0.02 at T_6) (Table 3.1; Figure 3.4).

At T_1, T_4, T_5, and T_6, the proportion of *H. coagulans* SNZ1969® in vegetative form was higher than that in spore form, but a significant difference was observed at T_4, 20 minutes after the start of chewing (3.3±0.6 log10 CFU/mL vs 2.3±1.2 log10 CFU/mL; p=0.01) (Table 3.1; Figure 3.4).

A high variability in the salivary counts among participants was observed for all the probiotic strains tested (Figure 3.5).

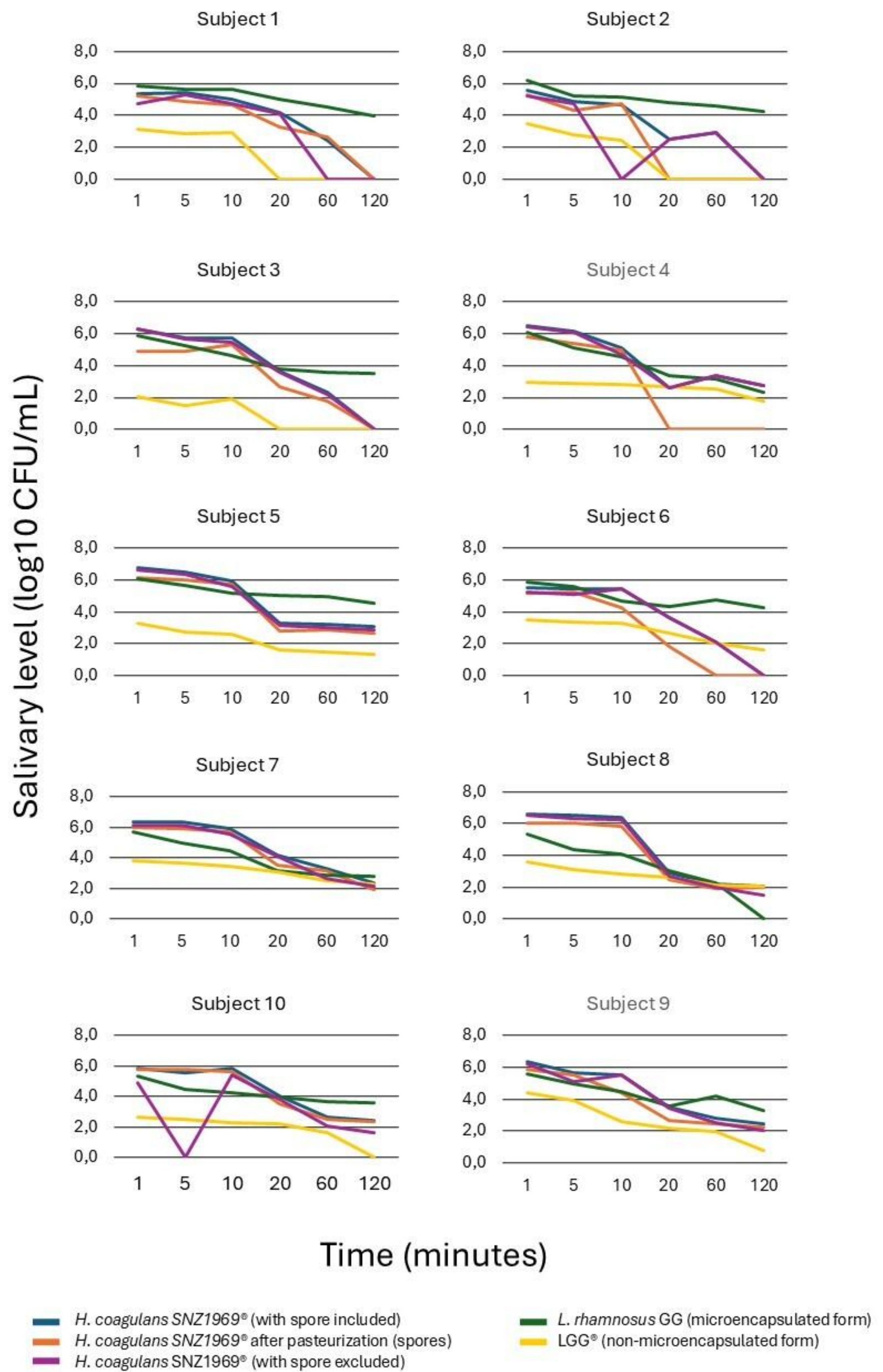

Figure 3.5. Viable count kinetics of probiotic cells in salivary samples of each subject.

All probiotic strains exhibited significant declines in salivary counts from T_1 to T_6 ($p<0.01$) (Table 3.1). Notably, the patterns differed between *L. rhamnosus* GG and *H. coagulans* SNZ1969®. For both microencapsulated and non-microencapsulated forms of *L. rhamnosus* GG, the counts differences between consecutive time points were not statistically significant, indicating a consistent linear decrease (Table 3.1). In contrast, total counts of *H. coagulans* SNZ1969® (counts of vegetative forms + spores) and its pasteurized form (spore counts) showed a rapid decline between T_3 and T_4 ($p<0.01$). The total counts of *H. coagulans* SNZ1969® also exhibited a significant drop between T_5 and T_6 ($p<0.01$) (Table 3.1). However, no significant differences were observed at each time point for *H. coagulans* SNZ1969® with spores excluded (Table 3.1).

Considering the area under the curve, a significant difference was detected between *L. rhamnosus* LGG® in non-microencapsulated form, *L. rhamnosus* GG in microencapsulated form and *H. coagulans* SNZ1969® (i.e., spores + vegetative cells) ($p<0.01$). Otherwise, significant differences between *L. rhamnosus* LGG® and *H. coagulans* SNZ1969® (i.e., spores + vegetative cells) were not detected ($p=0.90$) (Figure 3.6).

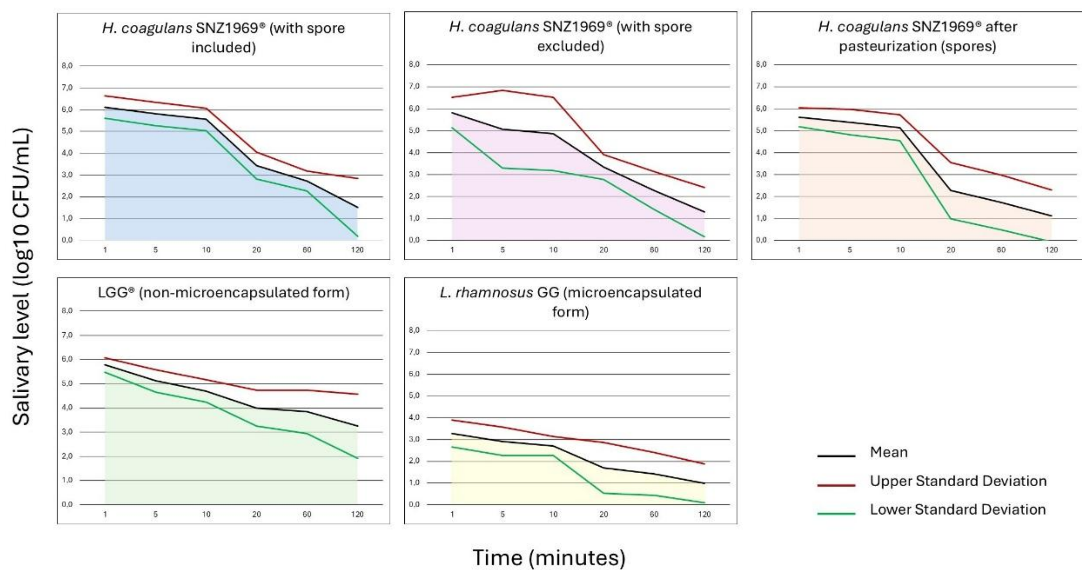

Figure 3.6. Area under the curve of probiotic cells in salivary samples.

3.4 Discussion

This viable microbiological study investigates the kinetics of *L. rhamnosus* GG and *H. coagulans* SNZ1969® released from sugar-free chewing gums. Despite considerable variability in salivary counts among participants, the highest bacterial amount was attained after one minute of chewing, with levels persisting at a relatively high counts for up to 10 minutes. In some subjects, probiotics were still detectable in saliva approximately two hours after chewing commenced.

Although there is a paucity of research in this area, studies have been conducted on the oral health benefits of probiotics administered via chewing gum. However, these studies have not specifically examined the kinetics of probiotics in saliva, focusing on their effects on oral biofilm, such as reducing S. mutans counts in saliva or dental plaque (87,98).

Few studies have evaluated the kinetics of probiotics obtained with different vehicles. Lozenges were shown to expose the oral environment to probiotics for an estimated period of tens of minutes (87,99). The utilization of a mucoadhesive lipogel comprising probiotics resulted in a substantial and consistent release for five to eight hours in an in vitro model (100). In the present study, chewing gum appears to promote the presence of probiotics in saliva for a longer time than lozenges but shorter than those obtained by lipogel in vitro. As anticipated, the probiotic load diminished following the gum mastication, yet it remained quantifiable in most subjects two hours post-ingestion. This comparatively protracted residence time in the oral environment may play a pivotal role in plaque colonization by the delivered bacteria.

Active agents administered via sugar-free chewing gum exhibited analogous kinetic trends in saliva. The investigation focused on the salivary concentration of xylitol and fluoride produced by sugar-free chewing gum (47,101), which showed a salivary peak in the first 5 minutes, followed by a rapid decrease in the next 5 minutes and a slow decline in the tens of minutes to follow. The salivary counts of the probiotics tested in this study initially peaked analogous to that of xylitol and fluoride. However, this was followed either by a gradual decrease, as seen with *L. rhamnosus* GG, or by a later but rapid decline (after 10

minutes), as observed with *H. coagulans* SNZ1969®. The decline in probiotic counts is mainly due to swallowing, which is intensified by the increased salivary flow stimulated by chewing gum. Additionally, the potential bactericidal action of salivary molecules cannot be ruled out (42,43,45,102).

The significant variability observed in the salivary counts of the probiotics among the ten study participants is a matter of concern. Despite the inclusion criterion of normal salivary flow, it can be hypothesized that such substantial inter-individual differences are not related to the efficacy of chewing gum in releasing probiotic strains. Instead, they are likely to be attributable to the individual characteristics of the participants, such as the strength and number of chewing movements per unit of time (103). Additionally, the different stimulation of saliva among subjects caused by the fruit flavouring in the chewing gum could further explain the observed variability (104).

One aspect that has been the focus of academic inquiry is microencapsulation's protective effect on chewing gum's bacterial survival. The study revealed that microencapsulation significantly enhanced the survival of *Limosilactobacillus reuteri* in chewing gum over 21 days. The protective role of inulin and lecithin in this process has been postulated (105). In a recent study, microencapsulation improved the viability of *Bifidobacterium animalis subsp. lactis*, but not of *Levilactobacillus brevis* (100,106). Although the encapsulated form may offer advantages in terms of microbial survival within the product, the results of the present study indicate that *L. rhamnosus* GG in its microencapsulated form produced a lower salivary count than the non-microencapsulated form, and by far the lowest among the probiotics tested. This finding suggests that the enhanced survival observed in the chewing gum product is counterbalanced by a diminished capacity to release the strain in its non-microencapsulated form. Consequently, probiotics in microencapsulated form may be advantageous for gastrointestinal effects but may not be as effective for promoting oral health.

There are certain limitations to this study. The first limitation is the limited sample size, which limits the generalisability of the results to a larger population (107,108). Probiotic studies designed and conducted for gastrointestinal application usually had a low number of participants as they focus solely on the

microbiological performance of the probiotic strain, independent of any direct effects on the host. The present research aligns with this established approach, as its primary objective was to quantify probiotic cells' presence in saliva after chewing gum administration. Given this specific focus, the sample size is fully consistent with existing literature. Numerous published works in this domain have employed similar sample sizes, as the primary endpoint (bacterial recovery) does not require the large cohorts typically associated with clinical efficacy trials. Furthermore, participants were selected according to rigorous criteria (e.g., number of teeth, salivary flow, and oral health status) to minimize uncontrolled variability, and the crossover design enhances the reliability of the results of this study by allowing within subject comparisons, thereby reducing inter-individual variability (106).

In the present study, it was ensured by verifying that none of the participants had detectable counts of the tested probiotic strains in their saliva before chewing the gum (T0 time point). A control group using a probiotic-free chewing gum would not provide additional meaningful information in this context, as this study does not aim to evaluate clinical effects or host responses but rather the presence of the administered probiotic in saliva. The presence or absence of probiotics in post-administration samples can be directly attributed to the Intervention itself, as confirmed by the absence of these strains in baseline samples.

Further research involving larger cohorts is necessary to draw definitive conclusions regarding the kinetics of probiotics released from sugar-free chewing gum, as this factor may significantly influence their functionality within the oral environment. Furthermore, the considerable variability observed in the counts of probiotics among the study participants requires further investigation, as it could substantially impact their efficacy. The present research shows that some individuals displace bacteria more quickly than others, underscoring the necessity for personalized approaches when contemplating probiotic Interventions, as individual differences can influence the effectiveness of such treatments.

3.5 Conclusion

This study demonstrated that a 10-minute use of sugar-free chewing gum containing probiotics led to their detectable presence in saliva. *L. rhamnosus* GG and *H. coagulans* SNZ1969® exhibited comparable salivary counts, suggesting that both strains are well-suited for this mode of administration.

It's well known that a single administration of probiotics is not capable of permanently modifying the composition of the oral bacterial flora (109). Further investigations are needed to determine the ideal duration of the probiotic's presence in the oral cavity to support colonization of the oral biofilm, thus improving its long-term efficacy.

4. Study III – Assessing the Impact on dental biofilm of *H. coagulans* administered through chewing gum: a double-blind randomized controlled trial

4.1 Aim

In the oral cavity, the role of *H. coagulans* has been studied to antagonize the proliferation of S. mutans, reducing the incidence of caries (72–76); moreover, there are some studies that have shown its beneficial effect in maintaining the health of the periodontium (71,78)and reducing the healing time of aphthous ulcers (77).

In this context, this randomized, double-blind, placebo-controlled trial evaluating *H. coagulans* SNZ1969® delivered via sugar-free chewing gum provides an opportunity to address two linked questions of translational relevance: (i) can the strain be detected and quantified in dental plaque during routine dosing and after cessation, and (ii) does its administration induce reproducible shifts in plaque community?

4.2 Material and Methods

Design of the study

The present randomized controlled trial aims to evaluate the ability of the probiotic *H. coagulans*, administered through sugar-free chewing gum, to colonize dental plaque. The study was designed and conducted at the Department of Biomedical, Surgical and Dental Sciences, University of Milan (Milan, Italy), between September 2024 and April 2025, in accordance with the principles of the Declaration of Helsinki. Ethical approval was obtained from the Ethics Committee of the University of Milan (February 13, 2024; protocol no. 24/24).

Sample selection

The study was conducted on healthy adult volunteers selected from the staff of the Perfetti Van Melle SpA (PVM, Lainate, Italy) and from students attending the degree in Dental Hygiene and in Dentistry and in post-degree of Pediatric Dentistry of University of Milan (Milan, Italy).

No prior studies with the same objective were identified, so a sample size calculation could not be performed. Accordingly, a sample size of 20 subjects per group was decided arbitrarily as reported in studies with analogous experimental designs (110) and increased to 52 subjects to outweigh for potential dropouts. The inclusion criteria were: adult subjects aged 18 to 64 years, at least 24 natural teeth (excluding third molars), gingival index and plaque index scores ≤ 2, and a stimulated salivary flow rate between 1.5 and 2.0 mL/min. Exclusion criteria included: the presence of systemic diseases, pregnancy or lactation, history of drug abuse, smoking habits, use of fixed orthodontic appliances, and allergies to any ingredients in the chewing gums used. Brochures providing a concise overview of the study objectives and participation procedures were displayed near the lecture halls and in the break room to recruit potential participants. A total of 56 individuals responded to the recruitment call. Two participants refused enrolment owing to challenges in ensuring adherence to the study protocol. Subjects who consented to participate were interviewed to assess their eligibility based on the inclusion and exclusion criteria. They were then examined by a calibrated dentist (SC) to obtain their gingival index scores (92), gingival index (93) and stimulated salivary flow rate. Two participants were excluded due to a potential allergy to the chewing gum components. Finally, fifty-two eligible subjects were identified and enrolled. Participants were randomized into two groups (26 in Intervention group and 26 in control group) using a computer-generated randomization system. Both participants and investigators were blinded to group allocation. All study participants gave their written consent to participate.

Chewing gums production

The Intervention chewing gums used in the Intervention group were produced as the chewing gum contained *Heyndrickxia coagulans* SNZ1969 used in Study III.

The control chewing gum was matched to the test gum in terms of shape, colour, and composition, and was produced using the same process, but it did not contain any probiotics.

Use of chewing gum

The study lasted a total of seven weeks. All enrolled subjects received instructions for at-home oral hygiene, along with a manual toothbrush and a fluoride toothpaste (1450 ppm F) to be used throughout the study period. Participants were instructed not to use any mouthwash or other oral hygiene products aside from those provided during the experimental phase. Additionally, subjects were asked to refrain from using antibacterial or antibiotic medications (either topical or systemic), from taking probiotics, and from consuming chewing gum or other products containing xylitol. Should the use of any of these products have been necessary, participants were required to notify the investigators and would subsequently be excluded from the study sample.

The experimental period was structured as follows: an initial two-week washout phase, followed by a four-week Intervention phase, and concluding with a one-week post-Intervention phase. During the four-week Intervention, participants were instructed to consume the assigned chewing gum five times per day (after breakfast, mid-morning, after lunch, mid-afternoon, and in the evening after dinner), at least 30 minutes after brushing their teeth.

To encourage compliance and proper product intake, participants were provided with blister packs containing the exact number of chewing gums to be consumed between two consecutive follow-up visits (n = 70). They were asked to bring the empty blister pack to the next visit to verify regular consumption. Additionally, participants were given a paper diary and instructed to record each chewing gum intake. Adherence to the protocol, at-home oral hygiene procedures, and any potential adverse effects were monitored using a custom-designed questionnaire, administered at each evaluation.

Follow-up assessments were conducted at the following time points: after the initial two-week washout period (T_0), after two weeks of chewing gum use (T_1), after four weeks of chewing gum use (T_2), and at the end of the post-Intervention period (T_3).

The primary outcome assessed was the characterization of the dental plaque microbial ecosystem. The secondary outcome was qPCR-based detection of *H. coagulans* SNZ1969® in dental plaque in subjects who received the probiotic-containing chewing gum.

DNA extraction from dental plaque samples and qPCR analyses

Approximately 1 mL samples of dental plaque were collected from the buccal and lingual surfaces of all teeth using sterile swabs (FLOQSwabs®, Copan Italia spa, Brescia, Italy). The samples were then placed in tubes containing 1 mL of nucleic acid collection and preservation medium (eNAT®, Copan Italia spa, Brescia, Italy). Samples were stored at 4° C, transported to the laboratory, and processed within two hours and immediately transferred to −80 °C until analysis.

Total DNA was extracted from dental plaque samples using the QIAsymphony DSP Virus/Pathogen Midi Kit® (Qiagen, Milan, Italy), following the manufacturer's instructions. DNA concentration was determined fluorometrically with a Qubit 4 Fluorometer® (Thermo Fisher Scientific, Segrate, Italy), and 10 ng of template DNA from each sample was used per real-time quantitative PCR (qPCR) reaction to detect and quantify *H. coagulans* SNZ1969® cells. Strain-specific primers described by Perotti et al. (111) were used: Wc 1F (5′-TTGTCTTTGGATCAGTTACAG-3′) and Wc-1R (5′-GCATAGGAATACCTTGTGCA-3′ (112), targeting a putative PimA-like glycosyltransferase (phosphatidyl-myo-inositol mannosyltransferase) gene, yielding a 192 bp amplicon. Each qPCR reaction (15 µL final volume) contained 7.5 µL EvaGreen® Supermix (Bio-Rad Laboratories, Segrate, Italy) and 0.5 µM of each primer. Thermal cycling conditions were: 98 °C for 30 s, followed by 40 cycles of 96 °C for 2 s and 59 °C for 5 s. Fluorescence was recorded at the end of each 59 °C step, and amplification specificity was verified by post-run melt-curve analysis. Cell-equivalent counts were calculated from a standard curve generated with DNA extracted from a pure culture of the target strain. Under detection limit (u.d.l.) has been determined at < 1.3 Log_{10} cells/ng.

Metataxonomic analysis via 16S rRNA gene profiling

DNA extracted from dental plaque, as described above, was processed and sequenced on an AVITI platform using paired-end 2×300 bp chemistry at the Center for Omics Sciences (COSR), San Raffaele Hospital (Milan, Italy). The provider delivered raw reads in FASTQ® format, run-level quality reports (FastQC®), and a technical report. The resulting sequences were analyzed to characterize bacterial community composition and assign taxonomic identities following the workflow outlined in Figure 4.1

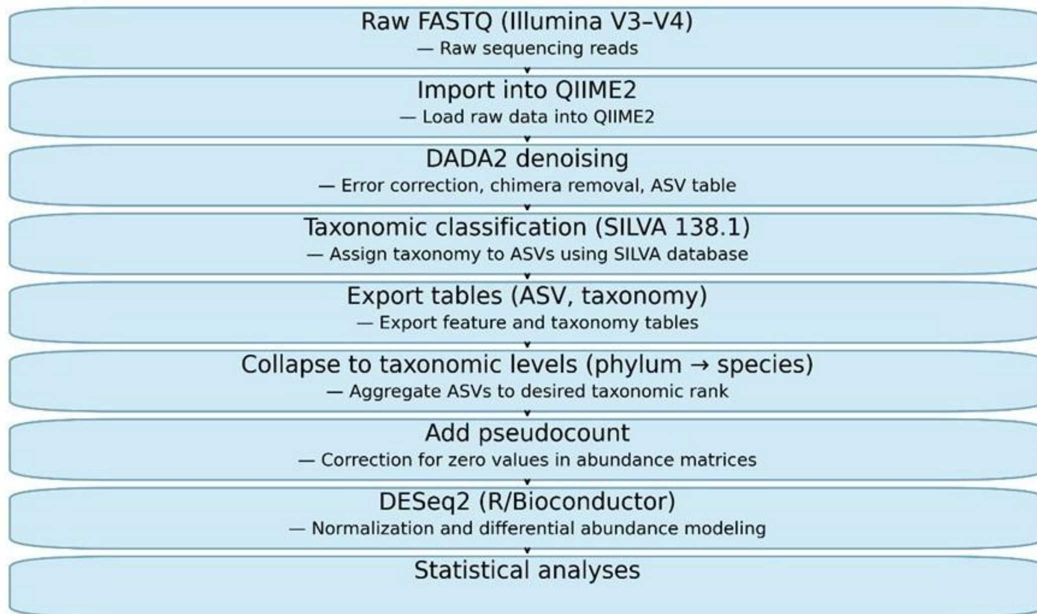

Figure 4.1. Schematic representation of the adopted bioinformatic workflow for 16S rRNA gene amplicon sequencing analysis.

Raw paired-end FASTQ® files were processed in QIIME 2™ (version 2024.5, q2-amplicon distribution. Caporaso Lab, Northern Arizona University, Flagstaff, AZ, USA). After demultiplexing, reads were quality-filtered, denoised, merged, and chimera-checked with the DADA2® plugin, generating an amplicon sequence variant (ASV) feature table and representative sequences. Alpha- and beta 6 diversity metrics were computed from the ASV table using the q2-diversity plugin. Specifically, within-sample diversity was assessed by observed features (richness), Faith’s phylogenetic diversity (Faith’s PD), Pielou’s evenness, and Shannon entropy. Between-sample diversity was evaluated using four dissimilarity metrics: weighted UniFrac, unweighted UniFrac, Jaccard, and Bray–Curtis. Taxonomic classification of ASVs was performed with a Naive Bayes classifier trained on SILVA release 138 (99% OTUs, 515F–806R region).

Abundances were summarized using taxa bar plots and exported at multiple taxonomic levels (from ASV to phylum). Inferential differential-abundance analysis was conducted with DESeq2 (Bioconductor) on raw counts aggregated by rank (phylum, class, order, family, genus), considering taxa present in ≥25% of samples (see paragraph describing statistics for more details).

Figure 4.2 summarizes the chewing gum administration schedule and the sample collection timeline

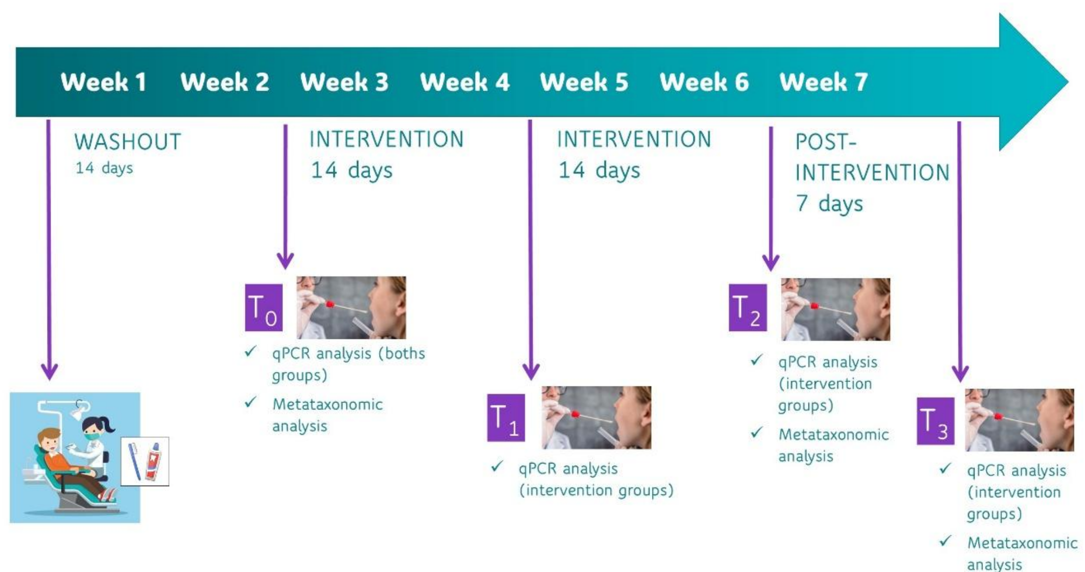

Figure 4.2 Chewing gum administration schedule and sample collection timeline.

Statistical analysis

To assess the Time × Treatment effect on alpha-diversity indices, we applied a rank-based "difference-in differences" strategy:

1. For each subject and metric, paired deltas were computed (T_2−T_0, T_3−T_0, and T_3− T_2).
2. Delta distributions were compared between Probiotic and Control groups using two-sided Mann Whitney U tests (MWU), the non-parametric analogue of an interaction test when time is expressed as paired differences.

3. Within-group temporal changes were assessed with two-sided Wilcoxon signed-rank tests on raw values for each group (Control, Probiotic) and time pair.

4. For MWU, effect sizes were reported as rank-biserial correlation (r); median deltas were also reported for both groups.

5. Multiple testing across contrasts was controlled using the Benjamini–Hochberg false discovery rate (FDR; q-values).

Analyses were performed in Python (SciPy/NumPy/Pandas; Matplotlib for visualization). For beta-diversity metrics, the first two principal coordinates (PC1 and PC2) were extracted and displayed in scatter plots stratified by treatment (Control vs. Probiotic) and time point (T_0, T_2, T_3). Temporal trajectories for each subject were visualized as arrows connecting time points in chronological order ($T_0 \rightarrow T_2 \rightarrow T_3$). Statistical differences across time points were assessed using two complementary approaches: (i) pairwise Mann-Whitney U tests for PC1 and PC2 between T_0 vs. T_2, T_1 vs. T_3, and T_2 vs. T_3 within each group; and (ii) Analysis of Similarities (ANOSIM, 999 permutations) on Euclidean distance matrices derived from PC1 and PC2, both for global comparisons (T_0, T_2, T_3) and pairwise contrasts.

For inferential differential-abundance analysis of bacterial taxa, a negative binomial generalized linear model with design formula Time × Treatment was fitted. Normalization was performed by estimating size factors with the median-of-ratios method, and dispersions were estimated per taxon according to the standard DESeq2 implementation. For interaction terms, $\log_2$ fold-changes and confidence intervals were estimated, and significance was tested with Wald tests. P-values were adjusted for multiple comparisons using the Benjamini-Hochberg procedure; results with $q < 0.05$ were considered significant. Analyses were performed separately at each taxonomic level and consolidated for presentation by reporting, for each taxon × time point pair, the most informative contrast.

4.2 Results

A total of 52 volunteers (26 per group) started the 2-week washout period. Three participants in the Intervention group were excluded, one for non-

compliance with the washout instructions, two owing to health complications that emerged after enrolment. Subsequently, 23 participants in the Intervention group and 26 in the Control group began chewing gum administration. During the study, one participant in the Control group dropped out due to dislike of the gum's taste, while one participant in the Intervention group withdrew because of gastro-intestinal disorders and were therefore excluded from the first follow-up (T_1). Before the second follow-up (T_2), two additional participants in the Placebo group were excluded: one discontinued chewing gum due to gastro-intestinal disorders, and another missed the follow-up for personal reasons. Ultimately, 21 participants in the Intervention group and 23 in the Control group completed the study (drop out 13.5%). Figure 4.3 and Table 4.1 resume recruitment, randomization, and follow-up of participants in the clinical trial.

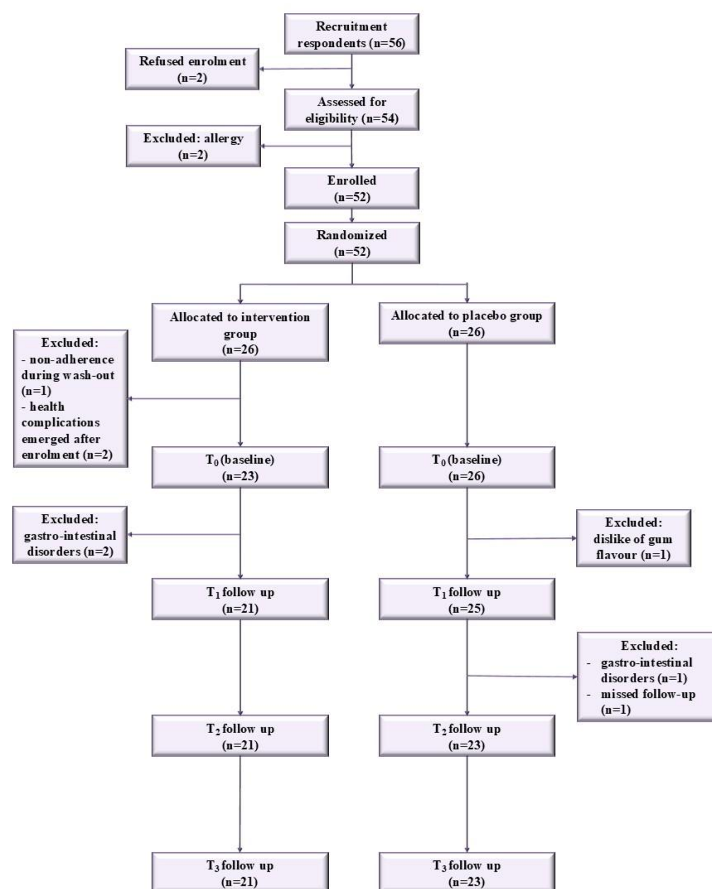

Figure 5.3. Flow diagram of participant recruitment, randomization, and follow-up in the clinical trial.

Table 5.1. Participant-level data from a clinical trial, detailing demographics, group allocation, protocol adherence, and reported adverse effects across multiple time points.

ID	Age (years)	Gender	Group	START WASH-OUT	T_0	Compliance (number of chewing gum skipped)	Unpleasant flavour, texture, and size of chewing gum	Gastro-intestinal disorders (pain/ bloating/ constipation / reflux)	T_1	Compliance (number of chewing gum skipped)	Unpleasant flavour, texture, and size of chewing gum	Gastro-intestinal disorders (pain/ bloating/ constipation / reflux)	T_2	T_3
1	50	F	Placebo	x	x	3	x	x	x				x	x
2	24	F	Placebo	x	x				x				x	x
3	48	M	Intervention	x	x		x		x				x	x
4	26	F	Intervention	x	x			x	x		x		x	x
5	37	F	Intervention	x	x	2		x	x	10			x	x
6	53	M	Placebo	x	x				x			x	x	x
7	39	F	Placebo	x	x			x	x			x	x	x
8	29	M	Intervention	x	x		x		x	1	x	x	x	x
9	51	F	Intervention	x	x			x	x				x	x
10	30	F	Intervention	x	x		x		x			x	x	x
11	Refused enrolment owing to challenges in ensuring adherence to the study protocol													
12	27	F	Intervention	x	x			x	x				x	x
13	26	F	Placebo	x	x		x		x				x	x
14	28	F	Placebo	x	x	x			Excluded (dislike of the chewing gum flavour)					
15	27	F	Placebo	x	x	2	x	x	x	3		x	x	x
16	Excluded due to a potential allergy to the chewing gum components													
17	24	F	Placebo	x	x				x				x	x
18	23	M	Placebo	x	x				x				x	x
19	24	F	Placebo	x	x				x				x	x
20	23	F	Intervention	x	x	2			x				x	x

21	22	F	Intervention	x	x				x	4			x	x
22	26	f	Intervention	x	Excluded due to non-adherence during the wash-out period									
23	28	F	Intervention	x	x			x	Excluded (g-i disorders)					
24	27	F	Placebo	x	x	4			x	4			x	x
25	29	F	Intervention	x	x				x	4			x	x
26	55	F	Intervention	x	x	2			x				x	x
27	28	F	Placebo	x	x				x				x	x
28	24	F	Placebo	x	x				x				x	x
29	29	F	Placebo	x	x				x				x	x
30	21	M	Placebo	x	x				x				x	x
31	20	M	Placebo	x	x	5			x				x	x
32	21	F	Intervention	x	x				x	5			x	x
33	Refused enrolment owing to challenges in ensuring adherence to the study protocol													
34	22	F	Intervention	x	x			x	x		x	x	x	x
35	Excluded due to a potential allergy to the chewing gum components													
36	25	F	Placebo	x	x				x	Excluded (missed follow-up)				
37	21	F	Intervention	x	x				x				x	x
38	20	F	Placebo	x	x				x				x	x
39	21	F	Intervention	x	x				x				x	x
40	20	F	Placebo	x	x				x			x	Excluded (g-i disorders)	
41	20	F	Intervention	x	x				x				x	x
42	22	F	Placebo	x	x				x				x	x
43	21	F	Intervention	x	x				x				x	x
44	45	F	Intervention	x	x			x	x			x	x	x
45	29	F	Placebo	x	x				x				x	x
46	23	F	Placebo	x	x				x				x	x
47	26	F	Placebo	x	x			x	x				x	x
48	24	F	Intervention	Excluded owing to health complications that emerged after enrolment										
49	24	F	Placebo	x	x			x	x				x	x
50	27	M	Intervention	x	x			x	Excluded (g-i disorders)					

51	23	F	Placebo	x	x				x				x	x
52	24	F	Placebo	x	x				x				x	x
53	23	F	Intervention	x	x				x				x	x
54	24	F	Intervention	Excluded owing to health complications that emerged after enrolment										
55	24	F	Intervention	x	x				x				x	x
56	23	F	Intervention	x	x				x				x	x

N: number; SD: standard deviation; M: male; F: female; g-i: gastro-intestinal.

The mean age of participants was 27.9 years (29.3±10.5 in the Intervention group and 27.0±8.2 in the Control group), and 85.7% were female (20 in the Intervention group and 22 in the Control group) (Table 4.2).

A total of 11 participants (7 in the Intervention group and 4 in the Control group) did not fully adhere to the chewing gum regimen; the mean of missed gums was 1.0±2.4/140 (range 0-12). Nine participants (5 in the Intervention group and 4 in the Control group) reported disliking the taste, texture, or size of the chewing gum. Furthermore, 17 participants (10 in the Intervention group and 7 in the Control group) reported gastrointestinal side effects, including bloating, reflux, gastritis, and abdominal pain. No statistically significant differences were found between groups (Table 4.2).

Table 4.2. Characteristics of participants, compliance and adverse effects reported in the two study groups during the experimental period

	Intervention N=23	Placebo N=26	Total N=49	p Value
Age (years)				
Mean (SD)	29.3 (10.5)	27.0 (8.2)	27.9 (9.0)	0.665[c]
Range	20.0;55.0	20.0;53.0	20.0;55.0	
Sex (n (%))				
F	20 (87.0)	22 (84.6)	42 (85.7)	1.000[a]
M	3 (13.0)	4 (15.4)	7 (14.3)	
Compliace				
N (%) of subject that skipped chewing gum during Intervention period	7 (30.4)	4 (15.4)	11 (22.5)	0.306[a]
Chewing gum skipped				
Mean (SD)	1.3 (2.8)	0.8 (2.1)	1.0 (2.4)	0.285[c]
Range	0.0;12.0	0.0;8.0	0.0-12.0	
Dislike (n (%) of subject that disliked chewing gum)	5 (21.7)	4 (15.4)	9 (18.4)	0.716[a]
Adverse effect (n (%) of subject that referred gastro-intestinal disorders)	10 (43.5)	7 (26.9)	17 (34.7)	0.224[b]

N: number; SD: standard deviation; M: male; F:f emale

Normality and hetereoskedasticity of continuous data were assessed with Shapiro-Wilk test. [a]Fisher's exact test; [b]Chi-square test; [c]Mann-Whitney U test.

Final analyses were conducted on the 44 subjects who completed the study (mean age: 29.48 in Intervention group and 27.39 in Control group). No statistically significant differences were observed in age or sex between

participants who used the probiotic chewing gum and those used the Placebo chewing.

Presence of H. coagulans in dental plaque samples

A total of 107 dental plaque samples (T_0 n=44; T_1 n=21; T_2 n=21; T_3 n=21) were analyzed by qPCR for the detection of strain *H. coagulans* SNZ1969. Values are expressed as $\log_{10}$ cell equivalents per ng of DNA ($\log_{10}$ cells/ng). The limit of detection (LOD) was 1.3 $\log_{10}$ cells/ng; values below the LOD are reported as under detection limit (u.d.l.). In the Control group (n=23), no sample was positive at T_0 (0/23). In the Intervention group (n=21), one volunteer was weakly positive at T_0 (1/21; 4.8%). During the Intervention, positivity was observed in 71.4% of subjects at T_1 (15/21) and 61.9% at T_2 (13/21); overall, 16/21 (76.2%) were positive at least once across T_1–T_2, and 12/21 (57.1%) at both time points. One week after discontinuation (T_3), positivity persisted in 2/21 subjects (9.5%; subject ID 26 and 37), while the others were u.d.l. All five non-responders (8, 25, 34, 39, 56) remained u.d.l. at T_1–T_3. Among positives, the target load showed median values of 2.3 $\log_{10}$ cells/ng (IQR 2.0–2.6; range 1.4–4.8) at T_1 and 2.4 (2.0–2.9; 1.6–5.1) at T_2. At T_3, the two subjects still positive exhibited high loads (median 4.4; range 4.2–4.7 $\log_{10}$ cells/ng). The most marked individual dynamics were observed in subjects 32 (1.4→4.8 log10 cells/ng from T_1 to T_2) and 37 (1.6→4.8→5.1→4.2 from T_0 to T_3), whereas 26 increased from 2.0 to 4.2 and then 4.7 across T_1, T_2, and T_3, respectively. Analytical specificity is supported by the absence of signal in controls and concordant melting curves. The results described above are reported in Table 4.3 and Figure 4.4.

Table 4.3. qPCR-based detection of the probiotic strain of *H. coagulans* in oral samples on subjects in Intervention group. Values are reported as Log_{10} of target-gene copies (cell equivalents) per ng of extracted DNA (Log_{10} cells/ng). u.d.l., under detection limit (< 1.3 Log_{10} cells/ng).

ID	T0	T1	T2	T3
3	u.d.l.	**2.88**	**2.68**	u.d.l.
4	u.d.l.	**2.44**	**2.03**	u.d.l.
5	u.d.l.	**2.27**	**1.63**	u.d.l.
8	u.d.l.	u.d.l.	u.d.l.	u.d.l.
9	u.d.l.	**2.73**	**2.94**	u.d.l.

10	u.d.l.	u.d.l.	**1.67**	u.d.l.
12	u.d.l.	**2.21**	**2.25**	u.d.l.
20	u.d.l.	**2.31**	**2.59**	u.d.l.
21	u.d.l.	**2.35**	**2.08**	u.d.l.
25	u.d.l.	u.d.l.	u.d.l.	u.d.l.
26	u.d.l.	**2.06**	**4.21**	**4.66**
32	u.d.l.	**1.42**	**4.75**	u.d.l.
34	u.d.l.	u.d.l.	u.d.l.	u.d.l.
37	**1.58**	**4.75**	**5.12**	**4.2**
39	u.d.l.	u.d.l.	u.d.l.	u.d.l.
41	u.d.l.	**3.00**	u.d.l.	u.d.l.
43	u.d.l.	**2.17**	u.d.l.	u.d.l.
44	u.d.l.	**1.88**	**2.38**	u.d.l.
53	u.d.l.	**1.62**	u.d.l.	u.d.l.
55	u.d.l.	**1.87**	**1.93**	u.d.l.
56	u.d.l.	u.d.l.	u.d.l.	u.d.l.

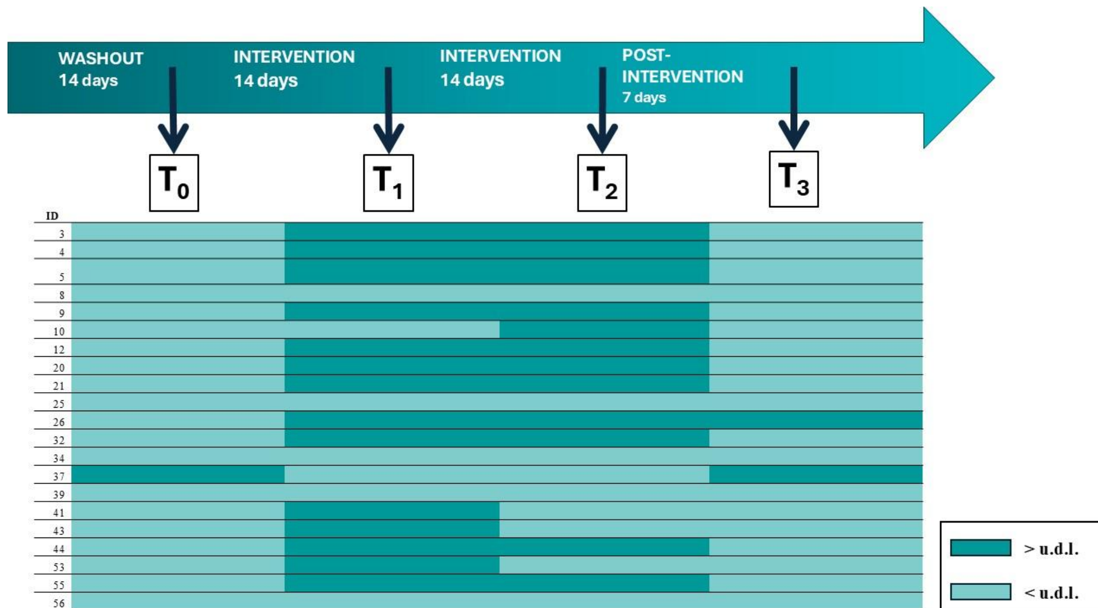

Figure 4.4. qPCR-based detection levels of the probiotic strain *H. coagulans* at different follow up points of subjects in Intervention group. u.d.l., under detection limit (< 1.3 Log_{10} cells/ng).

Analysis of the bacterial community structure of dental plaques: Alpha-diversity

In the longitudinal analysis of dental plaque alpha-diversity metrics, the Time × Treatment interaction, evaluated non-parametrically (Mann–Whitney U on deltas), showed during the Intervention ($T_0 \rightarrow T_2$) a significant divergence between Intervention and Control groups for Faith's phylogenetic diversity

(p=0.0027; n_control=24, n_probiotic=22; r=−0.52): the median Δ increased in the Control group (+1.68) and decreased in the Intervention group (−2.48). For Pielou's evenness, a between-group difference was observed (p=0.0160; r=+0.42), with median Δ ≈ −0.0026 in the Control and +0.0367 in the Intervention; accordingly, evenness increased within the Intervention group (Wilcoxon p=0.0103) but not within the Control (p=0.944). Faith's PD also changed within both groups over $T_0 \rightarrow T_2$ (Control p=0.0340; Intervention p=0.0275), albeit in opposite directions. No treatment dependent differences emerged for observed features or Shannon entropy, nor in the $T_0 \rightarrow T_3$ or $T_2 \rightarrow T_3$ contrasts for any metric. After FDR correction across all tests, however, no result reached q<0.005 (minimum q=0.0967 for Faith $T_0 \rightarrow T_2$); the signals observed over $T_0 \rightarrow T_2$ should therefore be interpreted as exploratory/hypothesis-generating. The results of these analyses are reported in Table 4.4 and Figure 4.5.

Table 4.4. Statistical analysis of alpha-diversity metrics in dental plaque across study groups and timepoints. Pairwise comparisons were performed using Mann–Whitney U tests (MWU) on deltas (between-group, Placebo vs Intervention) and Wilcoxon signed-rank tests (within-group, paired across timepoints). Reported are the number of subjects per group (n), raw p-values, and false discovery rate (FDR)-adjusted q-values (Benjamini–Hochberg correction applied across all tests). Significant results at $p < 0.05$ are highlighted in bold in the text, although none survived FDR correction ($q < 0.05$).

Variable	Comparison	Test	p_value	n	q_value FDR_BH
observed_features	**T_0-T_2**	**MWU (between-group deltas)**	**0.3170**		**0.7411**
observed_features	T_0-T_2	Wilcoxon (within Placebo)	0.2522	24	0.7411
observed_features	T_0-T_2	Wilcoxon (within Intervention)	0.6789	22	0.9397
observed_features	**T_0-T_3**	**MWU (between-group deltas)**	**0.1320**		**0.5421**
observed_features	T_0-T_3	Wilcoxon (within Placebo)	0.3596	24	0.7411
observed_features	T_0-T_3	Wilcoxon (within Intervention)	0.2756	22	0.7411
observed_features	**T_2-T_3**	**MWU (between-group deltas)**	**0.8604**		**0.9441**
observed_features	T_2-T_3	Wilcoxon (within Placebo)	0.7048	24	0.9397
observed_features	T_2-T_3	Wilcoxon (within Intervention)	0.5661	22	0.8860
faith_pd	**T_0-T_2**	**MWU (between-group deltas)**	**0.0027**		**0.0967**
faith_pd	T_0-T_2	Wilcoxon (within Placebo)	0.0340	24	0.2447
faith_pd	T_0-T_2	Wilcoxon (within Intervention)	0.0275	22	0.2447

faith_pd	**T_0-T_3**	**MWU (between-group deltas)**	**0.0969**		**0.5421**
faith_pd	T_0-T_3	Wilcoxon (within Placebo)	0.1355	24	0.5421
faith_pd	T_0-T_3	Wilcoxon (within Intervention)	0.4245	22	0.7457
faith_pd	**T_2-T_3**	**MWU (between-group deltas)**	**0.4885**		**0.7994**
faith_pd	T_2-T_3	Wilcoxon (within Placebo)	0.8553	24	0.9441
faith_pd	T_2-T_3	Wilcoxon (within Intervention)	0.3705	22	0.7411
pielou_evenness	**T_0-T_2**	**MWU (between-group deltas)**	**0.0160**		**0.1925**
pielou_evenness	T_0-T_2	Wilcoxon (within Placebo)	0.9441	24	0.9441
pielou_evenness	T_0-T_2	Wilcoxon (within Intervention)	0.0103	22	0.1858
pielou_evenness	**T_0-T_3**	**MWU (between-group deltas)**	**0.6208**		**0.8939**
pielou_evenness	T_0-T_3	Wilcoxon (within Placebo)	0.9218	24	0.9441
pielou_evenness	T_0-T_3	Wilcoxon (within Intervention)	0.3369	22	0.7411
pielou_evenness	**T_2-T_3**	**MWU (between-group deltas)**	**0.1834**		**0.6119**
pielou_evenness	T_2-T_3	Wilcoxon (within Placebo)	0.6033	24	0.8939
pielou_evenness	T_2-T_3	Wilcoxon (within Intervention)	0.1207	22	0.5421
shannon_entropy	**T_0-T_2**	**MWU (between-group deltas)**	**0.4350**		**0.7457**
shannon_entropy	T_0-T_2	Wilcoxon (within Placebo)	0.8553	24	0.9441
shannon_entropy	T_0-T_2	Wilcoxon (within Intervention)	0.1870	22	0.6119
shannon_entropy	**T_0-T_3**	**MWU (between-group deltas)**	**0.9212**		**0.9441**
shannon_entropy	T_0-T_3	Wilcoxon (within Placebo)	0.7683	24	0.9441
shannon_entropy	T_0-T_3	Wilcoxon (within Intervention)	0.7502	22	0.9441
shannon_entropy	**T_2-T_3**	**MWU (between-group deltas)**	**0.4222**		**0.7457**
shannon_entropy	T_2-T_3	Wilcoxon (within Placebo)	0.8996	24	0.9441
shannon_entropy	T_2-T_3	Wilcoxon (within Intervention)	0.3535	22	0.7411

MWU stands for Mann–Whitney U test (also known as the Wilcoxon rank-sum test). In practice, applying MWU to the deltas is the non-parametric equivalent of a Time × Treatment interaction: if the deltas differ between groups, this indicates a treatment effect on the temporal trajectory.

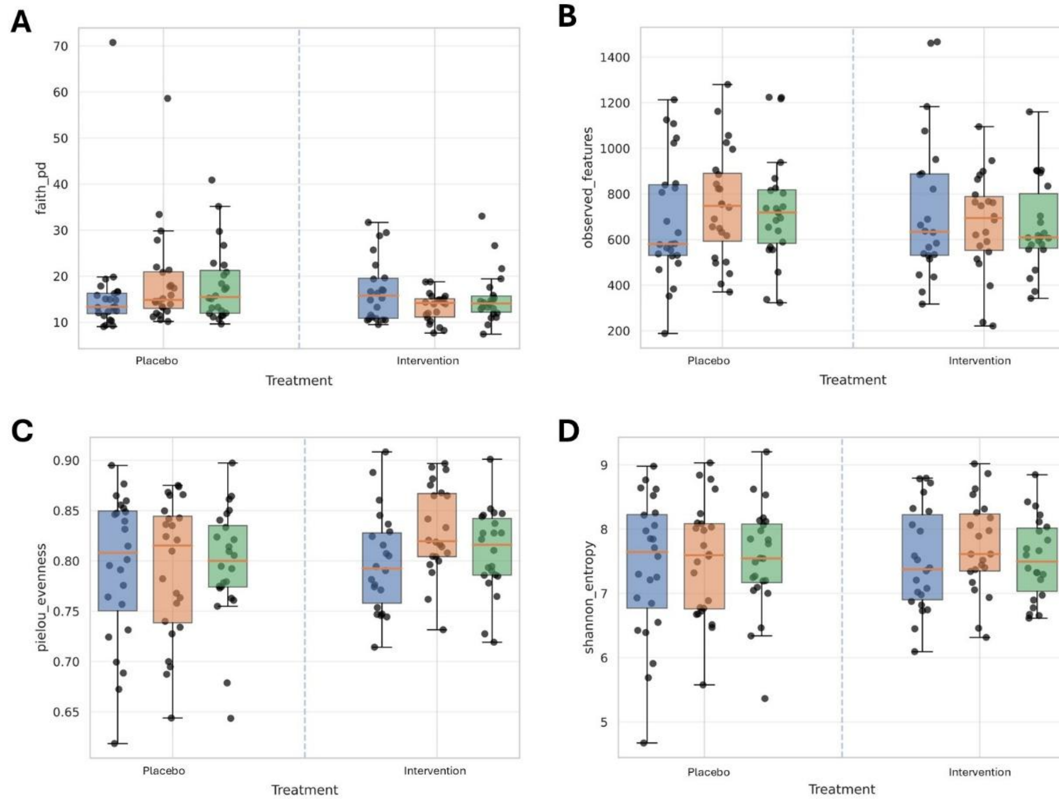

Figure 4.5. Alpha-diversity metrics of dental plaque microbiota across study groups and time points. Boxplots represent the distribution of (A) Faith's phylogenetic diversity, (B) observed features (richness), (C) Pielou's evenness, and (D) Shannon entropy in the Placebo and Intervention groups at baseline (T_0), end of Intervention (T_2), and follow-up (T_3). Each dot corresponds to an individual sample. Significant differences in deltas between groups were observed during the Intervention phase (T_0–T_2) for Faith's phylogenetic diversity ($p = 0.0027$) and Pielou's evenness ($p = 0.0160$), with the Intervention group showing increased evenness ($p = 0.0103$, Wilcoxon test). No significant group-dependent differences were detected for observed features or Shannon entropy.

Analysis of the bacterial community structure of dental plaques: Beta-diversity

Ordination plots showed substantial overlap among time points for both treatment groups and across all four beta-diversity metrics, with no clear clustering by time. Mann–Whitney U tests did not reveal significant differences between time points in either Control or Intervention groups (all $p > 0.05$). Some borderline trends were observed, including unweighted UniFrac in Control group (T_0 vs T_3, PC1, $p \approx 0.075$), unweighted UniFrac in Intervention group (T_0 vs T_2, PC1, $p \approx 0.125$), and Bray–Curtis in Intervention group (T_0 vs T_2, PC2, $p \approx 0.107$), but none reached significance (Table 4.5). ANOSIM

confirmed these findings: global comparisons yielded R values close to zero (wUniFrac R ≈ –0.027, p = 0.115; uwUniFrac R ≈ –0.012, p = 0.593; Jaccard R ≈ –0.036, p = 0.050; Bray–Curtis R ≈ –0.031, p = 0.075 in the Control group), indicating negligible group separation. Pairwise ANOSIM tests produced similarly low R values, with p-values > 0.05 in all cases (Table 4.6). Consistent with the statistical analyses, beta-diversity patterns are shown in Figure 4.6.

Table 4.5. Pairwise comparisons of beta-diversity principal coordinates among time points (T_0, T_2, T_3) in Control and Intervention groups. P-values were obtained using the non-parametric Mann–Whitney U test applied to the first two principal coordinates (PC1, PC2) derived from weighted UniFrac (wUniFrac), unweighted UniFrac (uwUniFrac), Jaccard, and Bray–Curtis dissimilarities. No comparison reached statistical significance ($p < 0.05$), although borderline values were observed for uwUniFrac (Placebo, T_0 vs T_3, PC1) and for Bray–Curtis (Intervention, T_0 vs T_2, PC2).

Method	**Treatment**	**Comparison**	**PC1_pvalue**	**PC2_pvalue**
wUniFrac	Placebo	T_0 vs T_2	0.792	0.930
wUniFrac	Placebo	T_0 vs T_3	0.629	0.660
wUniFrac	Placebo	T_2 vs T_3	0.982	0.709
wUniFrac	Intervention	T_0 vs T_2	0.669	0.258
wUniFrac	Intervention	T_0 vs T_3	0.920	0.900
wUniFrac	Intervention	T_2 vs T_3	0.763	0.131
uwUniFrac	Placebo	T_0 vs T_2	0.263	0.895
uwUniFrac	Placebo	T_0 vs T_3	0.075	0.913
uwUniFrac	Placebo	T_2 vs T_3	0.895	0.843
uwUniFrac	Intervention	T_0 vs T_2	0.125	0.960
uwUniFrac	Intervention	T_0 vs T_3	0.782	0.580
uwUniFrac	Intervention	T_2 vs T_3	0.258	0.960
Jaccard	Placebo	T_0 vs T_2	0.913	0.568
Jaccard	Placebo	T_0 vs T_3	1.000	0.826
Jaccard	Placebo	T_2 vs T_3	0.913	0.660
Jaccard	Intervention	T_0 vs T_2	0.513	0.314
Jaccard	Intervention	T_0 vs T_3	1.000	0.191
Jaccard	Intervention	T_2 vs T_3	0.529	0.725
bray_curtis	Placebo	T_0 vs T_2	0.809	1.000
bray_curtis	Placebo	T_0 vs T_3	0.583	0.895

bray_curtis	Placebo	T_2 vs T_3	0.758	0.982
bray_curtis	Intervention	T_0 vs T_2	0.880	0.107
bray_curtis	Intervention	T_0 vs T_3	0.880	0.651
bray_curtis	Intervention	T_2 vs T_3	0.940	0.191

Table 4.6. ANOSIM results for beta-diversity comparisons among time points (T_0, T_2, T_3) in Control and Intervention groups. Analyses were conducted on Euclidean distance matrices calculated from the first two principal coordinates (PC1 and PC2) derived from weighted UniFrac (wUniFrac), unweighted UniFrac (uwUniFrac), Jaccard, and Bray–Curtis dissimilarities. The table reports R statistics and permutation-based p-values (999 permutations) for global (T_0 vs T_2 vs T_3) and pairwise comparisons. All R values were close to zero or negative, indicating no meaningful separation between time points. Borderline trends were observed for Jaccard (Placebo, global comparison, $p = 0.050$) and Bray–Curtis (Placebo, global comparison, $p = 0.075$).

Method	**Treatment**	**Comparison**	**R_statistic**	**p_value**
wUniFrac	Placebo	Global (T_0,T_2,T_3)	-0.02659	0.1150
wUniFrac	Placebo	T_0 vs T_2	-0.01927	0.4240
wUniFrac	Placebo	T_0 vs T_3	-0.02375	0.3150
wUniFrac	Placebo	T_2 vs T_3	-0.03729	0.0920
wUniFrac	Intervention	Global (T_0,T_2,T_3)	-0.01680	0.5070
wUniFrac	Intervention	T_0 vs T_2	-0.01341	0.6900
wUniFrac	Intervention	T_0 vs T_3	-0.01885	0.5510
wUniFrac	Intervention	T_2 vs T_3	-0.01808	0.5900
uwUniFrac	Placebo	Global (T_0,T_2,T_3)	-0.01155	0.5930
uwUniFrac	Placebo	T_0 vs T_2	-0.01201	0.6860
uwUniFrac	Placebo	T_0 vs T_3	0.00774	0.7850
uwUniFrac	Placebo	T_2 vs T_3	-0.03033	0.2330
uwUniFrac	Intervention	Global (T_0,T_2,T_3)	-0.00192	0.9510
uwUniFrac	Intervention	T_0 vs T_2	0.02693	0.4040
uwUniFrac	Intervention	T_0 vs T_3	-0.02314	0.4790
uwUniFrac	Intervention	T_2 vs T_3	-0.01070	0.7450
Jaccard	Placebo	Global (T_0,T_2,T_3)	-0.03642	0.0500
Jaccard	Placebo	T_0 vs T_2	-0.03035	0.2030
Jaccard	Placebo	T_0 vs T_3	-0.04006	0.1090

Jaccard	Placebo	T_2 vs T_3	-0.03862	0.0860
Jaccard	Intervention	Global (T_0,T_2,T_3)	-0.02243	0.2510
Jaccard	Intervention	T_0 vs T_2	-0.02718	0.2940
Jaccard	Intervention	T_0 vs T_3	-0.01016	0.7350
Jaccard	Intervention	T_2 vs T_3	-0.03012	0.2450
bray_curtis	Placebo	Global (T_0,T_2,T_3)	-0.03068	0.0750
bray_curtis	Placebo	T_0 vs T_2	-0.03538	0.1300
bray_curtis	Placebo	T_0 vs T_3	-0.02676	0.2570
bray_curtis	Placebo	T_2 vs T_3	-0.03037	0.1530
bray_curtis	Intervention	Global (T_0,T_2,T_3)	-0.02001	0.3850
bray_curtis	Intervention	T_0 vs T_2	-0.01228	0.7180
bray_curtis	Intervention	T_0 vs T_3	-0.02507	0.4020
bray_curtis	Intervention	T_2 vs T_3	-0.02328	0.4740

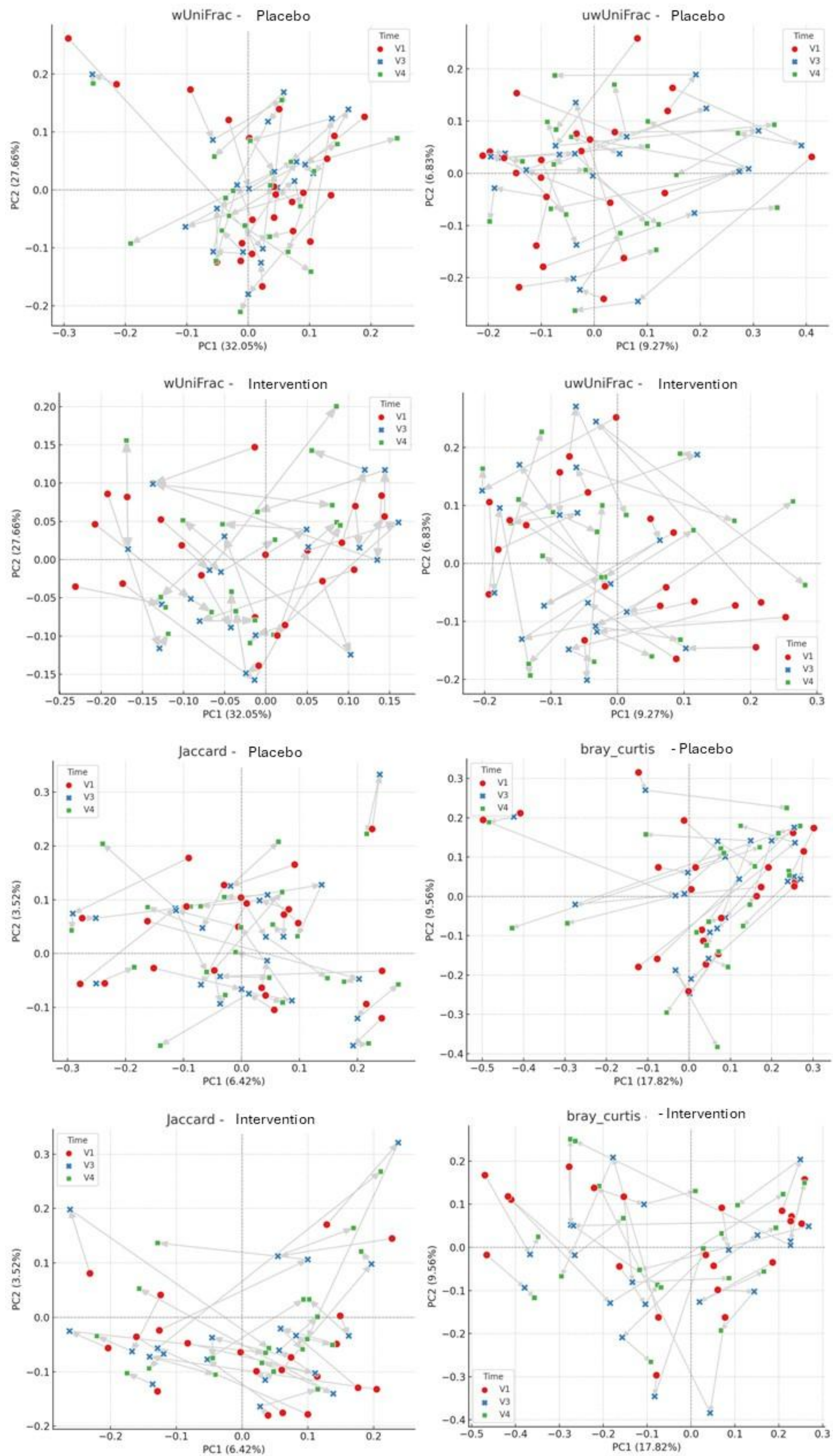

Figure 4.6. Principal coordinate analysis (PCoA) plots based on weighted UniFrac (wUniFrac), unweighted UniFrac (uwUniFrac), Jaccard, and Bray–Curtis dissimilarities. The

first two coordinates (PC1 and PC2) are shown, with the proportion of variance explained indicated on each axis. Samples are colored by time point (red=T_0, blue=T_2, green=T_3) and stratified by treatment group (Control and Intervention). Arrows connect longitudinal samples from the same subject ($T_0 \rightarrow T_2 \rightarrow T_3$), illustrating temporal trajectories. No clear clustering by time point was observed across either treatment group or beta-diversity metric.

Analysis of the bacterial community structure of dental plaques: Bacterial taxa

Using a DESeq2-like model with a Time × Treatment interaction, probiotic-associated shifts in dental plaque were identified, being most pronounced at the end of supplementation (T_2) and, more selectively, persisting at follow-up (T_3), thus highlighting taxa with divergent temporal trajectories between groups. At T_2, the probiotic group exhibited significant reductions across multiple lineages, including Actinobacteriota (orders Micrococcales, Propionibacteriales), Bacteroidota (orders Flavobacteriales and Sphingobacteriales, and Bacteroidales members such as Rikenellaceae_RC9_gut_group), Bacillota (orders Lactobacillales, Staphylococcales), Patescibacteria (order Saccharimonadales), and Pseudomonadota (orders Burkholderiales, Pasteurellales, Pseudomonadales); at lower ranks, decreases encompassed the families Micrococcaceae (genus Rothia), Carnobacteriaceae, Streptococcaceae, Staphylococcaceae, Gemellaceae, Weeksellaceae (genus Bergeyella), Lentimicrobiaceae (genus Lentimicrobium), Burkholderiaceae (genus Lautropia), Neisseriaceae (genus Neisseria), Pasteurellaceae, and Moraxellaceae. In contrast, the genus Lachnoanaerobaculum (member of the family Lachnospiraceae) increased in the Intervention group. At T_3, changes remained but were more selective, with sustained decreases in clinically relevant taxa including the genera Actinomyces (family Actinomycetaceae), Prevotella and Alloprevotella (Prevotellaceae), Rikenellaceae_RC9_gut_group, Tannerella (Tannerellaceae), Gemella (Gemellaceae), Leptotrichia (Leptotrichiaceae), Kingella (Neisseriaceae), Moraxella (Moraxellaceae), and Treponema (Spirochaetaceae), alongside reductions at higher ranks (families Lachnospiraceae, Neisseriaceae, Pseudomonadales, Saccharimonadaceae); concomitantly, an unclassified Actinomycetaceae genus (F0332) and Selenomonadaceae increased. The results of these analyses are reported in Table 4.7.

Overall, probiotic supplementation was associated with a decrease in the relative abundance of numerous oral taxa (with maximal effects at T_2 and still evident at T_3) and a selective enrichment of specific commensals or low-abundance taxa.

Table 4.7. Differentially abundant bacterial taxa between Intervention and Control groups. (A) Taxa significantly modulated at timepoint T_2 (end of probiotic treatment) according to DESeq2-like analysis. (B) Taxa significantly modulated at timepoint T_3 (follow-up after treatment discontinuation). The statistical model tested the interaction between time and treatment. Reported coefficients (Coef_log_rate) therefore represent the relative change in abundance of each taxon in the Intervention group compared to the change observed in the Control group at the same timepoint. A positive coefficient indicates that a taxon increased more (or decreased less) in the Intervention group than in the Control group, while a negative coefficient indicates the opposite pattern. The table shows the taxonomic classification, estimated log rate ratio (Coef_log_rate), FDR-adjusted q-value, and the direction of the effect.

A

Taxon	Coef_log_rate	qval	direction
p_Actinobacteriota;c_Actinomycetes;o_Micrococcales	-2.54	0.0020	↓ in Intervention
p_Actinobacteriota;c_Actinomycetes;o_Micrococcales;f_Micrococcaceae	-2.83	0.0000	↓ in Intervention
p_Actinobacteriota;c_Actinomycetes;o_Micrococcales;f_Micrococcaceae;g_*Rothia*	-1.47	0.0140	↓ in Intervention
p_Actinobacteriota;c_Actinomycetes;o_Propionibacteriales	-1.24	0.0490	↓ in Intervention
p_Bacteroidota;c_Bacteroidia;o_Bacteroidales;f_Rikenellaceae;g_Rikenellaceae_RC9_gut_group	-4.27	0.0010	↓ in Intervention
p_Bacteroidota;c_Bacteroidia;o_Flavobacteriales	-1.50	0.0040	↓ in Intervention
p_Bacteroidota;c_Bacteroidia;o_Flavobacteriales;f_Flavobacteriaceae	-1.38	0.0020	↓ in Intervention
p_Bacteroidota;c_Bacteroidia;o_Flavobacteriales;f_Flavobacteriaceae;g_*Capnocytophaga*	-1.47	0.0080	↓ in Intervention
p_Bacteroidota;c_Bacteroidia;o_Flavobacteriales;f_Weeksellaceae	-1.33	0.0310	↓ in Intervention
p_Bacteroidota;c_Bacteroidia;o_Flavobacteriales;f_Weeksellaceae;g_*Bergeyella*	-1.44	0.0010	↓ in Intervention
p_Bacteroidota;c_Bacteroidia;o_Sphingobacteriales;f_Lentimicrobiaceae;g_*Lentimicrobium*	-27.13[a]	0.0000	↓ in Intervention
p_Bacillota;c_Bacilli;o_Lactobacillales	-1.62	0.0080	↓ in Intervention
p_Bacillota;c_Bacilli;o_Lactobacillales;f_Carnobacteriaceae	-1.19	0.0490	↓ in Intervention
p_Bacillota;c_Bacilli;o_Lactobacillales;f_Streptococcaceae	-1.35	0.0370	↓ in Intervention
p_Bacillota;c_Bacilli;o_Staphylococcales	-1.65	0.0040	↓ in Intervention
p_Bacillota;c_Bacilli;o_Staphylococcales;f_Gemellaceae	-1.48	0.0050	↓ in Intervention
p_Bacillota;c_Clostridia;o_Lachnospirales;f_Lachnospiraceae;g_*Lachnoanaerobaculum*	1.86	0.0220	↑ in Intervention
p_Patescibacteria;c_Saccharimonadia;o_Saccharimonadales;f_Saccharimonadaceae;g_Candidatus_*Saccharimonas*	-27.05[a]	0.0000	↓ in Intervention
p_Pseudomonadota;c_Alphaproteobacteria;o_Rhizobiales;f_Xanthobacteraceae	-24.64[a]	0.0000	↓ in Intervention
p_Pseudomonadota;c_Gammaproteobacteria;o_Burkholderiales	-1.41	0.0040	↓ in Intervention
p_Pseudomonadota;c_Gammaproteobacteria;o_Burkholderiales;f_Burkholderiaceae	-2.69	0.0000	↓ in Intervention
p_Pseudomonadota;c_Gammaproteobacteria;o_Burkholderiales;f_Burkholderiaceae;g_*Lautropia*	-2.88	0.0000	↓ in Intervention
p_Pseudomonadota;c_Gammaproteobacteria;o_Burkholderiales;f_Neisseriaceae	-1.64	0.0030	↓ in Intervention

p_Pseudomonadota;c_Gammaproteobacteria;o_Burkholderiales;f_Neisseriaceae;g_*Neisseria*	-1.11	0.0350	↓ in Intervention
p_Pseudomonadota;c_Gammaproteobacteria;o_Pasteurellales	-1.94	0.0000	↓ in Intervention
p_Pseudomonadota;c_Gammaproteobacteria;o_Pasteurellales;f_Pasteurellaceae	-1.84	0.0070	↓ in Intervention
p_Pseudomonadota;c_Gammaproteobacteria;o_Pseudomonadales;f_Moraxellaceae[b]	-4.61	0.0050	↓ in Intervention

B

Taxon	Coef_log_rate	qval	direction
p_Actinobacteriota;c_Actinomycetes;o_Actinomycetales;f_Actinomycetaceae;g_*Actinomyces*	-2.13	0.0050	↓ in Intervention
p_Actinobacteriota;c_Actinomycetes;o_Actinomycetales;f_Actinomycetaceae;g_F0332	3.80	0.0120	↑ in Intervention
p_Bacteroidota;c_Bacteroidia;o_Bacteroidales;f_Prevotellaceae;g_*Alloprevotella*	-1.21	0.0230	↓ in Intervention
p_Bacteroidota;c_Bacteroidia;o_Bacteroidales;f_Prevotellaceae;g_*Prevotella*	-2.96	0.0220	↓ in Intervention
p_Bacteroidota;c_Bacteroidia;o_Bacteroidales;f_Rikenellaceae;g_Rikenellaceae_RC9_gut_group	-24.78[a]	0.0000	↓ in Intervention
p_Bacteroidota;c_Bacteroidia;o_Bacteroidales;f_Tannerellaceae;g_*Tannerella*	-25.91[a]	0.0000	↓ in Intervention
p_Bacillota;c_Bacilli;o_Staphylococcales;f_Gemellaceae;g_*Gemella*	-1.32	0.0370	↓ in Intervention
p_Bacillota;c_Clostridia;o_Lachnospirales;f_Lachnospiraceae	-1.43	0.0050	↓ in Intervention
p_Bacillota;c_Negativicutes;o_Veillonellales-Selenomonadales;f_Selenomonadaceae	2.10	0.0030	↑in Intervention
p_Bacillota;c_Negativicutes;o_Veillonellales-Selenomonadales;f_Selenomonadaceae;g_*Selenomonas*	-1.42	0.0350	↓ in Intervention
p_Fusobacteriota;c_Fusobacteriia;o_Fusobacteriales;f_Leptotrichiaceae;g_*Leptotrichia*	-2.89	0.0000	↓ in Intervention
p_Patescibacteria;c_Gracilibacteria;o_Absconditabacteriales_(SR1)	-1.38	0.0230	↓ in Intervention
p_Patescibacteria;c_Saccharimonadia;o_Saccharimonadales;f_Saccharimonadaceae;g_Candidatus_*Saccharimonas*	-27.99[a]	0.0000	↓ in Intervention
p_Pseudomonadota;c_Gammaproteobacteria;o_Burkholderiales;f_Neisseriaceae	-1.20	0.0310	↓ in Intervention
p_Pseudomonadota;c_Gammaproteobacteria;o_Burkholderiales;f_Neisseriaceae;g_*Kingella*	-1.52	0.0340	↓ in Intervention
p_Pseudomonadota;c_Gammaproteobacteria;o_Pseudomonadales	-6.34	0.0000	↓ in Intervention
p_Pseudomonadota;c_Gammaproteobacteria;o_Pseudomonadales;f_Moraxellaceae[b]	-5.23	0.0000	↓ in Intervention
p_Pseudomonadota;c_Gammaproteobacteria;o_Pseudomonadales;f_Moraxellaceae;g_*Moraxella*[b]	-7.15	0.0000	↓ in Intervention
p_Spirochaetota;c_Spirochaetia;o_Spirochaetales;f_Spirochaetaceae;g_*Treponema*	-26.11[a]	0.0000	↓ in Intervention

[a] these extreme Coef_log_rate values largely reflect the very low prevalence of such sparse taxa across samples, and should therefore be interpreted with caution.

[b] taxa detected in less than 25% of the samples.

4.4 Discussion

The aggregate qPCR data indicate that the administration of *H. coagulans* SNZ1969® via chewing gum led, in most subjects, to detectable levels of the bacterial DNA in dental plaque within 2 weeks, with positivity maintained in ~62% at 4 weeks and persisting in ~10% one week after discontinuation. The absence of signals at baseline (T_0) in the Control group and in 95% of subjects in the Intervention group suggests that positivity was attributable to the Intervention rather than pre-existing presence of the target bacterium. In our previous study, conducted with the same strain (*H. coagulans* SNZ1969®) delivered through the identical chewing gum formulation, persistence in saliva was assessed by plating and cultivation, and the strain was shown to remain detectable for at least 2 hours post-administration (111). Median loads during the Intervention were ~2.3-2.4 log10 cells/ng, with peaks exceeding 4.7–5.1, and individual trajectories (subject with ID 26, 32, 37) were consistent with genuine colonization or local accumulation of cells/spores in subsets of "responders". By contrast, 5/21 subjects remained consistently negative, pointing to inter-individual variability (e.g., adherence, oral physiology, dental plaque microecology) that warrants further investigation. Normalization per ng of total DNA reduced, but did not eliminate, variability due to sampling. Nonetheless, such inter-individual heterogeneity was noted also in previous oral probiotic clinical studies (111,113). It should be emphasized, however, that qPCR detects target DNA without distinguishing between live/active cells, spores, or free DNA; the term "colonization" should therefore be used cautiously in the absence of viability or metabolic evidence. Regarding alpha-diversity, during active administration ($T_0 \rightarrow T_3$), the Intervention group exhibited ecological trajectories distinct from Control group. Specifically, (i) Faith's PD decreased in the Intervention group while increasing in the Control group, and (ii) evenness increased in the Intervention group with minimal change in the Control group. The combination of reduced phylogenetic breadth and increased evenness suggests selective reshaping of community structure, limiting phylogenetic diversity while redistributing relative abundances more uniformly, as observed in other oral microbiome modulation studies (114,115).

Such a pattern is consistent with targeted competitive interactions and/or niche replacement, reducing dominance effects within the community. Importantly, no effects persisted to T_3 (post-cessation), indicating reversibility and dependence on continued exposure. However, although $T_0 \rightarrow T_2$ differences reached nominal significance ($p < 0.05$), they did not withstand FDR correction, underscoring the need for cautious interpretation and validation in larger, adequately powered cohorts. Overall, these findings suggest that probiotics delivered via chewing gum can transiently modulate dental plaque ecology, increasing evenness while altering phylogenetic composition, without durable effects after discontinuation. The functional and clinical implications of this diversity signature (lower Faith's PD with higher evenness) warrant further study, ideally linking taxonomic and functional profiles to oral health outcomes. For beta-diversity, no significant temporal changes were detected across any of the four metrics in either group. Both Mann-Whitney tests and ANOSIM consistently indicated no robust differences among time points (R statistics close to zero; non-significant p-values). Borderline trends (e.g., Jaccard and Bray–Curtis in 17 controls) were weak and likely reflect random variation rather than systematic changes. Thus, within the studied timeframe, the Intervention did not produce measurable shifts in dental plaque microbial structure at the beta diversity level, a pattern consistent with other RCTs showing stability in overall community structure despite transient probiotic effects (113,116). The 'DESeq2-like' differential analysis with a Time × Treatment interaction (subject included as a blocking factor) revealed divergent compositional trajectories between Intervention and Control groups, with a broad, acute suppression at T_2 (end of dosing) affecting orders/families typically associated with dysbiotic states, and a more selective persistence at T_3 (follow-up), featuring maintained decreases in lineages linked to periodontal disease and limited enrichments of potential commensals. Specifically, within the phylum Pseudomonadota (formerly Proteobacteria) we observed at T_2 coordinated reductions in Burkholderiales (including Burkholderiaceae and Lautropia), Pasteurellales/Pasteurellaceae, and Pseudomonadales/Moraxellaceae; at T_3 these were joined by decreases in Neisseriaceae (including Neisseria and Kingella) and in Pseudomonadales (with Moraxella). These groups include oral/respiratory opportunists or inflammation-associated colonizers, and their attenuation is consistent with competitive

exclusion, resource depletion, and/or microenvironmental remodeling induced by supplementation (115,117,118). Furthermore, it should be considered that Neisseria and Kingella, together with other genera such as Rothia, are key nitrate reducers. Therefore, it can be speculated that their decline may lower the overall capacity for nitrate → nitrite → NO conversion, with potential functional consequences. To determine whether this ecological shift is beneficial or entails a detrimental loss of function, microbiological analyses should be complemented by functional assessments, including salivary nitrate/nitrite levels and exhaled NO (119–121). Within phylum Bacteroidota (formerly Bacteroidetes) we found at V3 decreases in Flavobacteriales (Flavobacteriaceae, Weeksellaceae/Bergeyella) and Capnocytophaga and, at T_3, marked decreases in Prevotella/Alloprevotella, Tannerella, and Rikenellaceae_RC9. These genera/lineages are consistently associated with gingivitis and periodontitis, and the temporal comparison between the Intervention and the Control groups suggests that the probiotic either reduced the increases occurring in the Control group or reinforced the decreases, indicating a shift away from mature proteolytic anaerobic consortia (121–124). In phylum Bacillota (formerly Firmicutes), T_2 showed reductions in Staphylococcales (including Gemellaceae) and Lactobacillales (Carnobacteriaceae, Streptococcaceae), whereas at T_3 Gemella and the family Lachnospiraceae declined; in the opposite direction, Lachnoanaerobaculum increased at T_2, consistent with the saccharolytic/acidogenic nature of the genus and with possible transient niches opened by treatment, followed by family-level re-equilibration at T_3 (125,126). Among anaerobic “late colonizers” and “bridge” organisms, sustained T_3 decreases were recorded in Leptotrichia (Fusobacteriota) and Treponema (Spirochaetota), in line with an attenuation of mature/inflammatory consortia after supplementation (126–128). In phylum Actinobacteriota (formerly Actinobacteria), T_2 showed declines in Micrococcales/Micrococcaceae (Rothia) and Propionibacteriales (typical early colonizers of hard surfaces) while at T_3 Actinomyces decreased and an unclassified Actinomycetaceae (F0332) increased, indicating taxon specific selection rather than homogeneous phylum-level shifts; notably, Rothia is a known nitrate reducer, with 18 functional implications analogous to the note above on Neisseriaceae (129–131). For the candidate phyla radiation

(Patescibacteria/TM7), Saccharimonadaceae (including Candidatus Saccharimonas) showed consistently negative coefficients at T_2 and T_3; since TM7 interacts epibiotically/parasitically with Actinomyces and is implicated in periodontal inflammation, its sustained reduction is indicative of a potentially favorable microbial rebalancing. Nevertheless, caution is warranted because large apparent changes in low-abundance taxa can be strongly influenced by data sparsity (132,133). Overall, the pattern-broad suppression at T_2 followed by selective persistence at T_3 is consistent with targeted ecological remodeling of the bacterial biofilm, with depletion of proteolytic/anaerobic consortia (e.g., Prevotella/Tannerella/Treponema and various Pseudomonadota) and circumscribed rebounds of commensals (e.g., Lachnoanaerobaculum at T_2; Actinomycetaceae F0332 and Selenomonadaceae at T_3). This type of "targeted and modest-magnitude" effect aligns with clinical evidence on the use of probiotics as adjuncts in periodontal therapy (134,135).

LIMITATIONS - Methodologically, our log-linear approach mirrors DESeq2 in applying size-factor normalization and count-based inference, but it uses heteroskedasticity-robust standard errors to estimate the Time × Treatment interaction. However, the compositional structure of subject 16 data, together with potential heterogeneity within families or genera (for example, Selenomonadaceae increasing while Selenomonas decreases), highlights important limitations. These considerations argue for focusing on q-values and effect directions, taking taxon prevalence into account, and planning follow-up analyses at the ASV or strain level. In addition, shotgun metagenomics would be needed to achieve functional resolution. Within these constraints, interpreting the results in a difference-in-differences framework remains consistent with best practice for longitudinal repeated-measures microbiome studies (135–139).

4.5 Conclusions

In this randomized, Placebo-controlled study, qPCR experiments showed that *Heyndrickxia coagulans* SNZ1969® delivered via chewing gum reached detectable burdens in most treated volunteers during dosing (71.4% at 2 weeks; 61.9% at 4 weeks), with persistence in 9.5% one week after discontinuation; median loads were ~2.3–2.4 $\log_{10}$ cells/ng during Intervention and peaks exceeded 5 $\log_{10}$ cells/ng in responders, while all Control samples remained

negative, supporting effective release and local accumulation, albeit without proof of viability and with marked inter-individual variability. Metataxonomic analyses indicated a transient reshaping of alpha-diversity during Intervention (decrease in Faith's PD with a concomitant increase in evenness in the probiotic arm), no effects surviving FDR correction, and no significant time-dependent separation at the beta-diversity level. Difference-in-differences inference on taxa revealed a broad depletion at the end of probiotic administration (T_2), with selective persistence at follow-up (T_3), in lineages frequently implicated in oral dysbiosis/periodontitis (e.g., Prevotella/Alloprevotella, Tannerella, Treponema, Moraxellaceae/Moraxella, Neisseriaceae/Neisseria/Kingella, Leptotrichia), alongside limited enrichments of putative commensals (e.g., Lachnoanaerobaculum at T_2; Actinomycetaceae F0332 and Selenomonadaceae at T_3). Overall, the data support that *Heyndrickxia coagulans* SNZ1969® administered through chewing gum can modulate dental plaque ecology with modest and reversible community-wide effects and selective, potentially 19 favorable compositional shifts; confirmation in larger, adequately powered cohorts integrating viability assays, functional readouts (e.g., oral nitrate-reducing capacity), and clinically relevant endpoints is warranted.

5. Study IV - Chewing Gum added with *H. coagulans* on Halitosis: A Double-Blind, Randomized Controlled Trial

5.1 Aim

Although studies have explored the effects of *H. coagulans* on oral health conditions such as dental caries (72–76,78), gingivitis (78), and periodontitis (71) to date no study has specifically investigated its potential impact on halitosis. This gap in the literature is noteworthy, especially considering the increasing interest in probiotic-based interventions for managing oral malodour. Therefore, the aim of this study was to evaluate whether a chewing gum enriched with *H. coagulans* could effectively reduce halitosis by lowering levels of volatile sulphur compounds (VSCs), particularly hydrogen sulphide (H_2S) and methyl mercaptan (CH_3SH), in comparison to a placebo chewing gum.

5.2 Material and Methods

Design of the study

The present randomized controlled trial aims to evaluate the ability of the probiotic *H. coagulans* SNZ1969®, administered through sugar-free chewing gum, to modified VSCs in exhaled breath. The study was designed and conducted at the Department of Biomedical, Surgical and Dental Sciences, University of Milan (Milan, Italy), between September 2024 and April 2025, in accordance with the principles of the Declaration of Helsinki. Ethical approval was obtained from the Ethics Committee of the University of Milan (February 13, 2024; protocol no. 24/24).

Sample selection, production and use of chewing gums

Sample size, chewing gum production, use of chewing gum and follow up timeline were the same that in Study III.

Outcomes measures

The outcome assessed was the evaluation of volatile sulphur compounds (VSCs) released in the exhaled breath.

VSCs in exhaled breath were measured using a halimeter (OralChroma™, INSISTEC, Barcelona, Spai). All participants were tested between 8:00 and 10:00 a.m. Prior to testing, they were instructed to avoid the consumption of garlic, onion, and other strong-flavoured condiments, and to limit their intake of margarine, milk, fried foods, sardines, salami, mortadella, sausage, red meat, cheese, sulphur-containing foods (such as cabbage, broccoli, cauliflower, eggs), and alcohol on the day preceding the test. Additionally, participants were asked to refrain from consuming candies and chewing gum on the morning of the assessment. The values of H_2S and CH_3SH were considered due to their closer association with oral health, in line with previous studies (140–142). Halitosis was recorded when levels H_2S was ≥ 112 ppb and CH_3SH was ≥ 26 ppb.

Figure 5.1 summarizes the chewing gum administration schedule and the sample collection timeline.

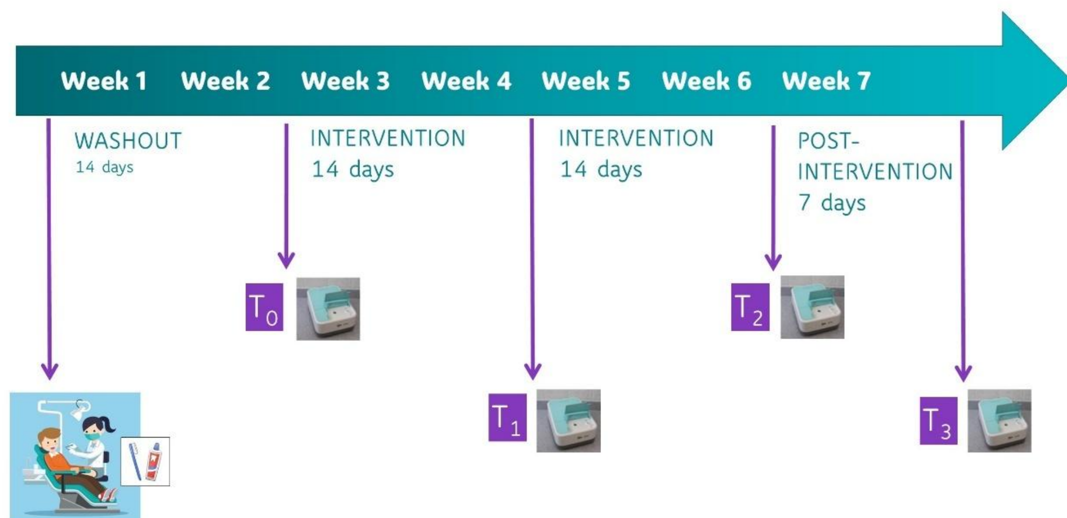

Figure 5.1. Chewing gum administration schedule and the sample collection timeline.

Statistical analysis

Descriptive statistics were calculated for all variables and reported as mean ± standard deviation (SD). The Shapiro–Wilk test was used to assess the normality and heteroskedasticity of continuous data. For between-group comparisons of continuous variables, the Mann–Whitney U test was applied due to non-normal data distributions. For categorical variables, Fisher's exact test was used to evaluate differences between groups. Intra-group comparisons of

repeated measures (T0, T1, T2, T3) were performed using the Wilcoxon signed-rank test for paired data. In specific subgroup analyses, Welch's t-test was applied when assumptions for equal variances were violated. A p-value < 0.05 was considered statistically significant. All statistical analyses were conducted using Stata/MP 19.0 for Windows.

5.3 Results

Demographic characteristics of the sample, drop out, side effect reported and compliance during the study were the same that in Study III.

Final analyses were conducted on the 44 subjects who completed the study. No statistically significant differences were observed in age or sex between participants who used the probiotic chewing gum and those using the placebo chewing gum (Table 5.1).

Table 5.1. Characteristics of subjects in the Intervention and Control groups, and VSCs (H_2S and CH_3SH) mean values at baseline (T_0), after 14 and 28 days from the beginning of administration (T_1 and T_2), and 7 days after the end of administration (T_3).

	Variable	Intervention N=21	Placebo N=23	Total N=44	p-Value
	Age	29.48 (10.71)	27.39 (8.39)	28.39 (9.62)	0.962[b]
	Gender				
	M	2 (9.52)	4 (17.39)	6 (13.6)	0.666[a]
	F	19 (90.48)	19 (82.61)	38 (86.4)	
Time	**VSCs** (ppb)				
T_0	H_2S	76.81 (94.34)	73.39 (124.54)	75.02 (111.17)	0.394[b]
	CH_3SH	28.05 (30.62)	22.65 (26.15)	25.23 (28.50)	0.787[b]
T_1	H_2S	53.67 (154.82)	66.74 (79.45)	60.50 (121.58)	0.070[b]
	CH_3SH	29.57 (56.98)	20.52 (25.55)	24.84 (43.72)	1.000[b]
T_2	H_2S	36.90 (87.16)	37.39 (57.51)	37.16 (73.17)	0.327[b]
	CH_3SH	27.29 (35.07)	25.26 (31.03)	26.23 (33.04)	0.787[b]
T_3	H_2S	67.62 (189.15)	43.17 (71.12)	54.84 (140.96)	0.868[b]
	CH_3SH	35.10 (65.40)	23.78 (34.64)	29.18 (51.96)	0.689[b]
p-Value[d]					
T_0 *vs* T_1	H_2S	0.121	0.637		
	CH_3SH	0.728	0.749		
T_0 *vs* T_2	H_2S	0.008*	0.088		
	CH_3SH	0.835	0.704		
T_0 *vs* T_3	H_2S	0.031*	0.437		
	CH_3SH	0.917	1.000		
T_1 *vs* T_2	H_2S	0.381	0.053		
	CH_3SH	0.972	0.939		
T_1 *vs* T_3	H_2S	0.714	0.025		

	CH_3SH	0.741	0.301
T_2 *vs* T_3	H_2S	0.754	0.626
	CH_3SH	0.931	0.903

N: number; SD: standard deviation; M: male; F: female
Normality and hetereoskedasticity of continuous data were assessed with Shapiro-Wilk test.
[a]Comparisons between groups were performed using Fisher's exact test.
[b] Comparisons between groups were performed using Mann-Whitney U test.
[d]Comparisons between groups were performed using Wilcoxon signed-rank test

At baseline, there were no statistically significant differences in H_2S and CH_3SH levels between the Intervention group and the Control group (Table 5.1).

The analysis of H_2S and CH_3SH concentrations, expressed in ppb, showed a progressive decrease in median values over time in both groups.

For H_2S, baseline levels (T_0) displayed wide variability in both groups, with several high outliers. At subsequent time points (T_1–T_3), a reduction in median concentrations was observed, which was more pronounced in the Intervention group. At T_2 and T_3, median values in the Intervention group were close to zero, with significant reductions compared with T_0 ($p = 0.008$ and $p = 0.031$, respectively), accompanied by a narrower dispersion compared with the Control group (Table 5.1; Figure 5.2 A).

Similarly, CH_3SH concentrations showed a downward trend from baseline to later time points. Although reductions were observed in both groups, the effect was more evident in the Intervention group, where median values at T_2 and T_3 were lower than those of the Control group. Variability also progressively decreased, suggesting a more consistent effect of the Intervention. In the intra-group comparison, no significant differences were observed in CH_3SH levels across the different time points, either in the Intervention group or in the Control group (Table 5.1; Figure 5.2 B).

Overall, these findings indicate that the probiotic produced a more substantial and sustained reduction in VSCs levels compared with placebo chewing gum, but there weren't significantly difference between the two groups (Table 5.1).

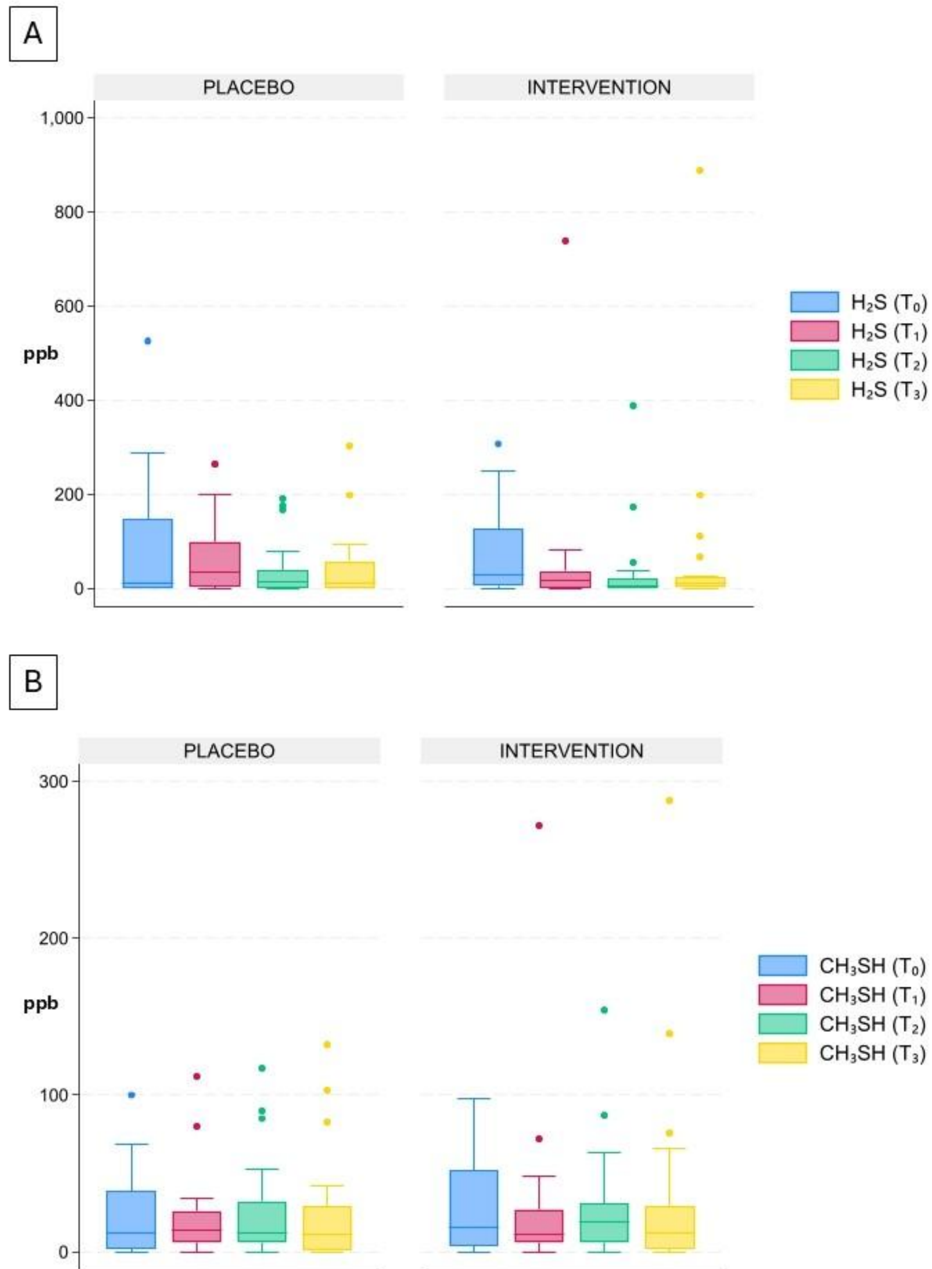

Figure 5.2. Box plots of hydrogen sulphide (H_2S) (A) and methyl mercaptan (CH_3SH) (B) concentrations (ppb) at baseline (T_0) and follow-up time points (T_1–T_3) in the Placebo and Intervention groups.

Twelve subjects (6 in the Intervention group and 6 in the Control group) presented H_2S levels above the halitosis threshold, while sixteen subjects (8 in the Intervention group and 8 in the Control group) showed CH_3SH levels above the threshold at baseline. Separate analyses were conducted for these subjects.

The results for subjects with elevated H_2S levels are summarized in Table 5.2.

In the Control group, median H_2S levels showed a progressive decline from T_0 to T_3, with the highest concentrations observed at baseline (T_0) and a marked reduction by T_2 and T_3. Variability among participants decreased over time, as indicated by the narrowing of the interquartile ranges (Table 5.2; Figure 5.3).

In the intervention group, H_2S levels exhibited a more complex pattern. While a decrease was observed at T_1 compared to T_0, levels partially rebounded at T_2 and T_3, with higher inter-individual variability, particularly at T_2 and T_3. Notably, several outliers with elevated H_2S concentrations were observed at T_1 and T_3. Overall, the intervention appeared to modulate H_2S concentrations differently than placebo, with greater fluctuations and outlier values over time (Table 5.2; Figure 5.3).

The within-group comparison showed a significant reduction in the mean H_2S value in the Control group between T_0 and T_2 ($p = 0.028$), T_0 and T_3 ($p = 0.046$), T_1 and T_2 ($p = 0.046$) and T_1 and T_3 ($p = 0.028$). No significant differences were found in the analyses conducted within the Intervention group (Table 5.2).

Table 5.2. Characteristics of subjects with H_2S levels above the halitosis threshold at baseline in the Intervention and Control groups, and H_2S mean values at baseline (T_0), after 14 and 28 days from the beginning of administration (T_1 and T_2), and 7 days after the end of administration (T_3).

Variable	Intervention N=6	Placebo N=6	Total N=12	p-Value
Age	28.00 (± 11.59)	25.17 (± 2.32)	26.58 (7.76)	0.686[b]
Gender				
M	0 (0.00)	1 (16.67)	1 (8.30)	>0.999[a]
F	6 (100.00)	5 (83.33)	11 (91.70)	
Time	**VSCs** (ppb)			
T_0	211.67 (63.65)	248.50 (131.96)	230.08 (105.22)	0.873[b]
T_1	151.00 (264.08)	148.83 (88.58)	149.92 (196.96)	0.109[b]
T_2	110.67 (136.07)	58.33 (57.83)	84.50 (107.77)	0.748[b]
T_3	218.50 (305.30)	60.00 (65.26)	139.25 (234.55)	0.229[b]
p-Value[d]				
T_0 *vs* T_1	0.345	0.116		
T_0 *vs* T_2	0.094	0.028*		
T_0 *vs* T_3	0.345	0.046*		
T_1 *vs* T_2	0.753	0.046*		
T_1 *vs* T_3	0.116	0.028*		
T_2 *vs* T_3	0.463	0.834		

N: number; SD: standard deviation; M: male; F: female

Normality and hetereoskedasticity of continuous data were assessed with Shapiro-Wilk test.
aComparisons between groups were performed using Fisher's exact test.
b Comparisons between groups were performed using Mann-Whitney U test.
dComparisons between groups were performed using Wilcoxon signed-rank test

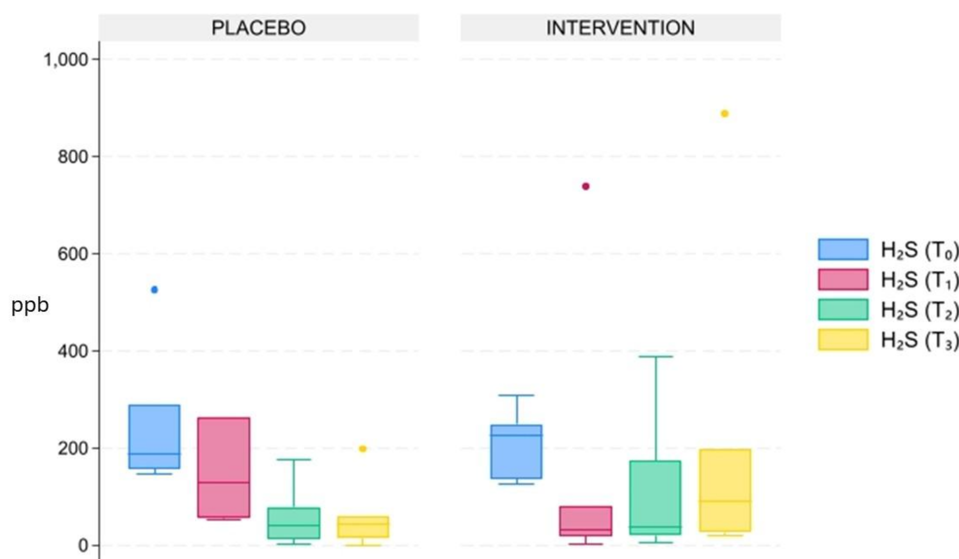

Figure 5.3. Box plots of hydrogen sulphide (H_2S) concentrations (ppb) of subjects with H_2S levels above the halitosis threshold at baseline (T_0) and follow-up time points (T_1–T_3) in the Placebo and Intervention groups.

In Table 5.3 summarize the results for subjects with elevated CH_3SH levels.

At baseline (T_0), CH_3SH levels were similar between groups. Over time, no consistent or marked reduction in CH_3SH concentration was observed in either group. Both Control and Intervention groups exhibited fluctuations in CH_3SH levels across the time points, with wide inter-individual variability. Notably, a few high outliers were observed at each time point, particularly at T_3 in the intervention group. Median values appeared relatively stable, and no significant differences were visually apparent between the groups or across time points ($p > 0.05$) (Table 5.3; Figure 5.4).

These findings suggest that the intervention did not lead to a clear or sustained reduction in CH_3SH levels compared to placebo during the study period.

Table 5.3. Characteristics of subjects with CH_3SH levels above the halitosis threshold at baseline in the Intervention and Placebo groups, and CH_3SH mean values at baseline (T_0),

after 14 and 28 days from the beginning of administration (T_1 and T_2), and 7 days after the end of administration (T_3).

Variable	Intervention N=8	Placebo N=8	Total N=16	p-Value
Age	34.00 (± 11.99)	24.50 (± 2.29)	29.25 (9.85)	0.076[b]
Gender				
M	1 (12.50)	2 (25.00)	3 (18.80)	>0.999[a]
F	7 (87.50)	6 (75.00)	13 (81.30)	
Time	**VSCs** (ppb)			
T_0	62.25 (22.12)	52.25 (23.14)	57.25 (23.18)	0.422[c]
T_1	57.75 (83.37)	25.63 (34.21)	41.69 (65.72)	0.227[b]
T_2	40.50 (47.06)	31.63 (26.89)	36.06 (38.58)	0.833[b]
T_3	80.13 (88.48)	49.13 (46.04)	64.63 (72.21)	0.462[b]
p-Value[d]				
T_0 *vs* T_1	0.326	0.124		
T_0 *vs* T_2	0.124	0.263		
T_0 *vs* T_3	0.889	0.779		
T_1 *vs* T_2	0.440	0.440		
T_1 *vs* T_3	0.206	0.483		
T_2 *vs* T_3	0.093	0.207		

N: number; SD: standard deviation; M: male; F: female
Normality and hetereoskedasticity of continuous data were assessed with Shapiro-Wilk test. [a]Comparisons between groups were performed using Fisher's exact test.
[b] Comparisons between groups were performed using Mann-Whitney U test.
[c] Comparisons between groups were performed using Welch's t test.
[d]Comparisons between groups were performed using Wilcoxon signed-rank test.

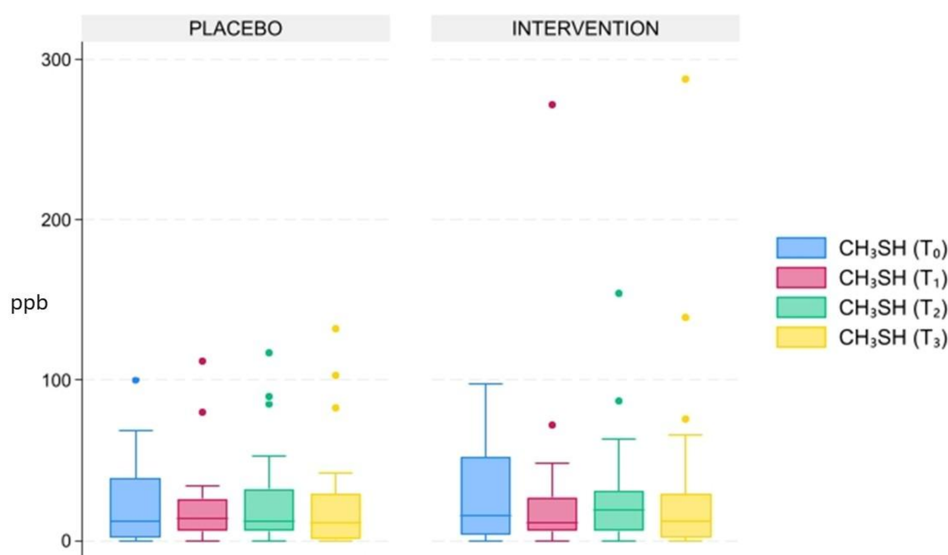

Figure 5.4. Box plots of hydrogen sulphide (CH_3SH) concentrations (ppb) of subjects with CH_3SH levels above the halitosis threshold at baseline (T_0) and follow-up time points (T_1–T_3) in the Placebo and Intervention groups.

5.3 Discussion

In this randomized, double-blind, placebo-controlled trial we investigated whether chewing gum enriched with *H. coagulans* could reduce levels of volatile sulfur compounds (VSCs), specifically hydrogen sulfide (H_2S) and methyl mercaptan (CH_3SH), compared with a placebo gum. Of the 44 participants who completed the study, those in the Intervention and Control groups were similar in age, sex, and baseline VSCs concentrations, which strengthens confidence that observed differences are attributable to the intervention rather than to demographic or baseline imbalances.

Over the course of the trial, both groups exhibited gradual declines in median concentrations of H_2S and CH_3SH. The reduction in H_2S was notably more pronounced in the Intervention group, particularly at the later time points (T_2 and T_3), where median values dropped close to zero. These reductions relative to baseline were statistically significant ($p = 0.008$ at T_2, $p = 0.031$ at T_3), and the spread of values narrowed, suggesting a consistent effect among many participants. By contrast, CH_3SH levels also trended downward, but changes were neither as large nor as consistent; within-group comparisons did not reach statistical significance, and reduction in the Intervention arm was not clearly distinct from placebo.

When focusing on participants who began the study with elevated VSCs levels, nuances emerged. Among those with high baseline H_2S in the Control group a significant decline from T_0 to later points was noted, with reductions evident at T_2 and T_3, and steady narrowing of variability. Curiously, in the Intervention group, the pattern was less straightforward: after an initial drop at T_1, H_2S levels partially rebounded at later points, and variability (including outliers) increased. Among subjects with elevated CH_3SH, neither group achieved consistent or statistically significant reductions. While median values fluctuated, large inter-individual differences persisted, and the Intervention did not produce a clear advantage.

These findings suggest that while *H. coagulans* gum may be effective in reducing H_2S, especially in the general population of participants, it may be less effective, or more variable in effect, for those with very high baseline H_2S, and

even less so for CH_3SH. Several mechanisms may account for these differential responses. Probiotic action may involve competition with sulphur-producing anaerobes for metabolic substrates, modulation of the biofilm environment (such as pH or redox potential), or production of inhibitory metabolites. Chewing gum itself also has salutary effects via increased saliva flow and mechanical removal of substrate; thus, improvements in both arms are expected to some degree.

Comparing with previous research, the present results align with a number of recent meta-analyses and randomized trials which find that probiotics more consistently reduce H_2S than CH_3SH. In meta-analyses of randomized clinical trials for oral halitosis, the majority of studies report significant reductions in total VSCs or in H_2S, whereas reductions in CH_3SH are often weaker or non-significant. For example, trials using *Lactobacillus salivarius*, *Weissella cibaria*, and *Streptococcus salivarius* strains have yielded better suppression of H_2S, or of organoleptic halitosis scores, than of CH_3SH (143). This study is distinguished by the use of H. coagulans in chewing gum form, a delivery mode that remains largely unexplored, particularly in longitudinal designs with multiple time points and subgroup analyses of individuals with elevated baseline VSCs.

That the Placebo group showed substantial declines in H_2S among high baseline subjects indicates that non-specific effects (e.g. gum chewing, increased awareness of oral hygiene, regression to the mean) can contribute to measurable improvements. This places a strong threshold for probiotic interventions to demonstrate added benefit. The fluctuating response in the Intervention group among high H_2S subjects may reflect heterogeneity in host factors, differences in oral microbiota composition, tongue coating, degree of anaerobic niches, diet, or saliva production, and perhaps limits in how much *H. coagulans* can suppress microbial activity in subjects with a high bacterial load or particularly aggressive sulfur-producing flora.

One plausible interpretation is that *H. coagulans* is more effective at suppressing or competing with bacteria that produce H_2S (which tend to be more broadly distributed and may rely on more accessible substrates), whereas CH_3SH producers, which often inhabit more protected, anaerobic sites (e.g.

subgingival or deep tongue fissures), are less accessible or less sensitive to suppression by topical probiotic delivered by chewing gum. Measurement limitations may also play a part: CH_3SH often is present in lower concentrations, may vary more over short times, or be more subject to environmental or dietary influences, making statistical detection of changes more difficult.

The gradual decline over time in both compounds suggests that duration of intervention matters: effects become more pronounced later (T_2). Shorter studies may miss these delayed responses (144). Similarly, in individuals with elevated initial VSCs, early improvements may be more modest or inconsistent, possibly because higher burden takes longer to reduce, or because of ceiling effects in certain behaviours or microbiome resilience.

Strengths of the study include the randomized and blinded design, use of repeated measures, measurement of specific VSCs rather than only summary scores, and good completion rates. The equivalence of baseline demographics and VSC levels adds to the internal validity. However, some limitations warrant discussion. The sample size, though adequate for detecting general trends, was modest for subgroup analyses, especially among subjects with elevated baseline compounds. This likely increased variability and reduced power to detect differences in CH_3SH, or in more nuanced patterns (145). Also, as is common in probiotic trials, the precise behaviour of *H. coagulans* in the oral cavity, colonization, persistence, interaction with other microbes, is not fully understood, which complicates causal inference. Measurement errors, or variability in VSC detection, may also have masked smaller effects. Moreover, behavioural or dietary factors, tongue coating, oral hygiene practices, and salivary flow were not exhaustively controlled or stratified, and could have contributed to inter-individual differences (145). Finally, the placebo gum itself has beneficial effects, which means the net advantage of the probiotic must be understood in that context rather than compared to doing nothing.

Altogether, this study indicates that chewing gum containing *H. coagulans* has potential as an adjunctive approach to managing halitosis, particularly for odour driven by hydrogen sulphide. While its effect on methyl mercaptan is less clear, the pattern of results, stronger and more consistent suppression of H_2S, especially at later time points, together with reduced dispersion among

participants, suggests clinically meaningful benefit for many users. For individuals with very high baseline H_2S, however, additional interventions (mechanical cleaning, tongue hygiene, perhaps higher or different probiotic dosing) may be necessary. Future investigations should involve larger sample sizes, longer follow-ups, and exploration of mechanisms, especially microbial community shifts, to understand who benefits most and under what circumstances.

5.4 Conclusion

In summary, this randomized controlled trial suggests that chewing gum containing *H. coagulans* produces a significant and sustained reduction in hydrogen sulphide levels in individuals relative to baseline, with more pronounced effects by mid- to late stages of the intervention. The effect on methyl mercaptan was less evident, with no statistically significant reductions in CH_3SH in either group, and marked inter-individual variability.

While the placebo gum also had some beneficial effects, particularly for H_2S in participants with elevated baseline levels, the probiotic intervention showed more consistent reduction and narrower dispersion in the overall sample. These findings align with existing literature that reports more robust effects of probiotics on H_2S than on CH_3SH, but they also reveal that efficacy may depend on baseline odor compound levels and individual variability.

Given the limitations, *H. coagulans* chewing gum holds promise as a complementary therapy for halitosis, particularly for odours dominated by hydrogen sulphide. However, further research is necessary to confirm these effects, optimize strain selection, dosage, delivery, and duration, and to identify subgroups that may benefit most.

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