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DINÂMICA DO ECOSSISTEMA ORAL NA DÍADE MÃE–BEBÊ: DO MICROBIOMA E DA PROTEÔMICA AO PERFIL IMUNOLÓGICO SALIVAR

Bragança Paulista
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DINÂMICA DO ECOSSISTEMA ORAL NA DÍADE MÃE–BEBÊ: DO MICROBIOMA E DA PROTEÔMICA AO PERFIL IMUNOLÓGICO SALIVAR

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Ferramentas de inteligência artificial (*ChatGPT, OpenAI*) foram utilizadas apenas para apoio linguístico e revisão gramatical, sob supervisão e edição integral dos autores.

"Você não pode esperar construir um mundo melhor sem melhorar os indivíduos. Para esse fim, cada um de nós deve trabalhar para o seu próprio aperfeiçoamento e, ao mesmo tempo, participar da responsabilidade coletiva por toda a humanidade."
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RESUMO

A gestação e os primeiros meses de vida constituem períodos críticos para o estabelecimento de hábitos e trajetórias biológicas que impactam a saúde ao longo da vida. Esta tese investigou, longitudinalmente, a saúde bucal materna, os perfis proteômico e imunológico salivares e a dinâmica do microbioma oral de gestantes/mães e seus respectivos bebês. Tal coorte foi acompanhada longitudinalmente desde o terceiro trimestre gestacional até seis meses pós-parto (CAAE: 65319322.2.0000.5514). A saúde bucal materna foi avaliada pelos índices CPOD e CPI, e informações socioeconômicas, antropométricas e alimentares foram obtidas por questionário. Amostras de saliva materna e infantil foram coletadas para análise proteômica por espectrometria de massas, quantificação de citocinas por ensaio multiplex e caracterização do microbioma oral por sequenciamento do gene 16S rRNA. Os dados foram analisados por estatística descritiva e bioinformática. De modo geral, as participantes apresentaram nível socioeconômico médio, elevada prevalência de cárie dentária (77,8%; CPOD 7,94) e alterações periodontais (≈60%), além de consumo frequente de bebidas adoçadas, como chá e café e carboidratos simples (35–40%). Após o nascimento, 60% dos bebês encontravam-se em aleitamento materno no 1º mês. A análise proteômica salivar identificou 21, 27 e 25 proteínas nos grupos de gestantes, mães e bebês, respectivamente, com cinco proteínas compartilhadas entre todos os grupos. A análise de vias biológicas evidenciou a presença recorrente de isoformas de alfa-amilase salivar (AMY1A/AMY1B/AMY1C) nos três grupos. A ontologia genética revelou similaridades funcionais entre gestantes e mães, especialmente quanto à atividade de alfa-amilase e à ligação a íons de cálcio, cloreto e metais, enquanto o grupo infantil apresentou predominância de processos relacionados ao metabolismo de carboidratos, oligossacarídeos e funções associadas à imunidade inata. Paralelamente, a caracterização do microbioma salivar revelou que a alfa-diversidade materna permaneceu estável ao longo do período avaliado e significativamente mais diverso ao dos bebês ($p<0,05$). Os microbiomas infantis apresentaram elevada variabilidade inicial e maior similaridade aos perfis maternos aos seis meses de vida (beta-diversidade, $p<0,05$). As comunidades iniciais foram dominadas por *Streptococcus*, seguidas por aumento de *Prevotella, Veillonella e Rothia*. Gêneros cariogênicos persistiram ao longo da infância, enquanto gêneros associados à periodontite, como *Aggregatibacter* e *Fusobacterium*, reduziram progressivamente e estavam ausentes aos seis meses. A análise de citocinas demonstrou perfis distintos entre mães e bebês, com diferenças consistentes em IL-8 e IL-12, além de variações dependentes do tempo em IFN-γ, IL-10, IL-13 e IL-1β. Esses achados indicam diferenças imunológicas salivares entre os grupos ao longo do acompanhamento longitudinal. Em conjunto, os achados demonstram uma sucessão ecológica dinâmica do microbioma salivar infantil, caracterizada por aumento progressivo da complexidade e maior similaridade em relação ao perfil materno ao longo dos primeiros seis meses de vida. Os achados são consistentes com o conceito do estabelecimento do microbioma oral considerando-se interações materno-infantis e exposições ambientais compartilhadas. A identificação de similaridades qualitativas nos perfis proteômicos maternos e infantis, e nos padrões imunológicos e microbianos, destaca potenciais alvos para estratégias de modulação de risco e promoção de saúde materno-infantil, especialmente no contexto de práticas alimentares e susceptibilidade a doenças bucais.

Palavras-chave: Gestante. Microbiota. Proteômica. Recém-Nascido. Saliva.

ABSTRACT

Pregnancy and the first months of life represent critical periods for the establishment of habits and biological trajectories that may influence health throughout the life course. This thesis longitudinally investigated maternal oral health, salivary proteomic and immunological profiles, and oral microbiome dynamics in pregnant/postpartum women and their respective infants. The cohort was followed from the third trimester of pregnancy through six months postpartum (CAAE: 65319322.2.0000.5514). Maternal oral health was assessed using the DMFT and CPI indices, and socioeconomic, anthropometric, and dietary information was obtained through questionnaires. Maternal and infant saliva samples were collected for proteomic analysis by mass spectrometry, cytokine quantification by multiplex assay, and oral microbiome characterization by 16S rRNA gene sequencing. Data were analyzed using descriptive statistics and bioinformatics. Overall, participants were from mid-level socioeconomic backgrounds and presented a high prevalence of dental caries (77.8%; DMFT = 7.94;) and periodontal alterations (≈60%), as well as frequent consumption of sweetened beverages, such as tea and coffee with sugar, and foods rich in simple carbohydrates (35–40%). After birth, 60% of infants were breastfed at one month of age. Salivary proteomic analysis identified 21, 27, and 25 proteins in the pregnant women, postpartum women, and infant groups, respectively, with five proteins shared among all groups. Biological pathway analysis revealed the recurrent presence of salivary alpha-amylase isoforms (AMY1A, AMY1B, and AMY1C) across the three groups. Gene ontology analysis demonstrated functional similarities between pregnant and postpartum women, particularly regarding alpha-amylase activity and calcium-, chloride-, and metal-ion binding, whereas the infant group showed a predominance of processes related to carbohydrate and oligosaccharide metabolism and functions associated with innate immunity. In parallel, salivary microbiome characterization demonstrated that maternal alpha-diversity remained stable throughout the follow-up period and was significantly higher than that observed in infants ($p<0.05$). Infant microbiomes exhibited high early variability, and progressively greater similarity to maternal profiles by six months of life (beta-diversity, $p<0.05$). Early microbial communities were dominated by *Streptococcus*, followed by increases in *Prevotella, Veillonella*, and *Rothia*. Cariogenic genera persisted throughout infancy, whereas periodontitis-associated taxa, such as *Aggregatibacter* and *Fusobacterium*, progressively declined and were absent at six months. Cytokine analysis demonstrated distinct profiles between mothers and infants, with consistent differences in IL-8 and IL-12 and time-dependent variations in IFN-γ, IL-10, IL-13, and IL-1β. These findings indicate differences in salivary immune profiles between groups throughout the longitudinal follow-up. Taken together, the findings demonstrate a dynamic ecological succession of the infant salivary microbiome, characterized by progressive increases in microbial complexity and greater similarity to the maternal profile during the first six months of life. The results are consistent with the concept of oral microbiome establishment within the context of maternal–infant interactions and shared environmental exposures. In addition, the identification of qualitative similarities in maternal and infant proteomic profiles, together with similar immunological and microbial patterns, highlights potential targets for risk-modulation strategies and maternal–infant health promotion, particularly in the context of dietary practices and susceptibility to oral diseases.

Keywords: *Pregnant Women. Microbiota. Proteomics. Infant. Saliva.*

LISTA DE SÍMBOLOS E ABREVIAÇÕES

AMY1A - Alfa-amilase salivar 1A

AMY1B - Alfa-amilase salivar 1B

AMY1C - Alfa-amilase salivar 1C

CAAE - Certificado de Apresentação para Apreciação Ética

CPI - Community Periodontal Index (Índice Periodontal Comunitário)

CPOD - Índice de dentes cariados, perdidos e obturados

IFN-γ - Interferon gama

IL-10 - Interleucina 10

IL-12 - Interleucina 12

IL-13 - Interleucina 13

IL-1β - Interleucina 1 beta

IL-8 - Interleucina 8

SB Brasil - Pesquisa Nacional de Saúde Bucal do Brasil

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1. INTRODUÇÃO

A gestação e a primeira infância são períodos marcados por intensas mudanças biológicas e comportamentais, nos quais hábitos de vida saudáveis podem ser estabelecidos, constituindo oportunidades estratégicas para intervenções de promoção da saúde. A boca abriga uma das maiores e mais diversificadas comunidades microbianas do corpo, e o estudo da microbiota na relação saúde-doença é fundamental para esclarecer como diferentes composições de microrganismos podem influenciar o equilíbrio entre estados de saúde e doença (PENG et al., 2022, NOVAES et al., 2026). Nesse contexto, duas doenças bucais mediadas por biofilme merecem destaque: a cárie dentária e a doença periodontal.

A doença periodontal compreende um conjunto de condições inflamatórias induzidas pelo biofilme, que afetam os tecidos de suporte dental (Figura 1). A gengivite corresponde à inflamação reversível restrita aos tecidos gengivais, enquanto a periodontite é caracterizada pela destruição progressiva do ligamento periodontal e do osso alveolar, levando à perda de inserção clínica e, em estágios avançados, à perda dentária (PIHLSTROM et al., 2005). A cárie dentária, por sua vez, é uma doença crônica, multifatorial e também biofilme-dependente, caracterizada pela desmineralização progressiva dos tecidos dentários em decorrência da produção de ácidos por microrganismos do biofilme a partir do metabolismo de carboidratos fermentáveis da dieta (Figura 1) (PITTS et al., 2017).

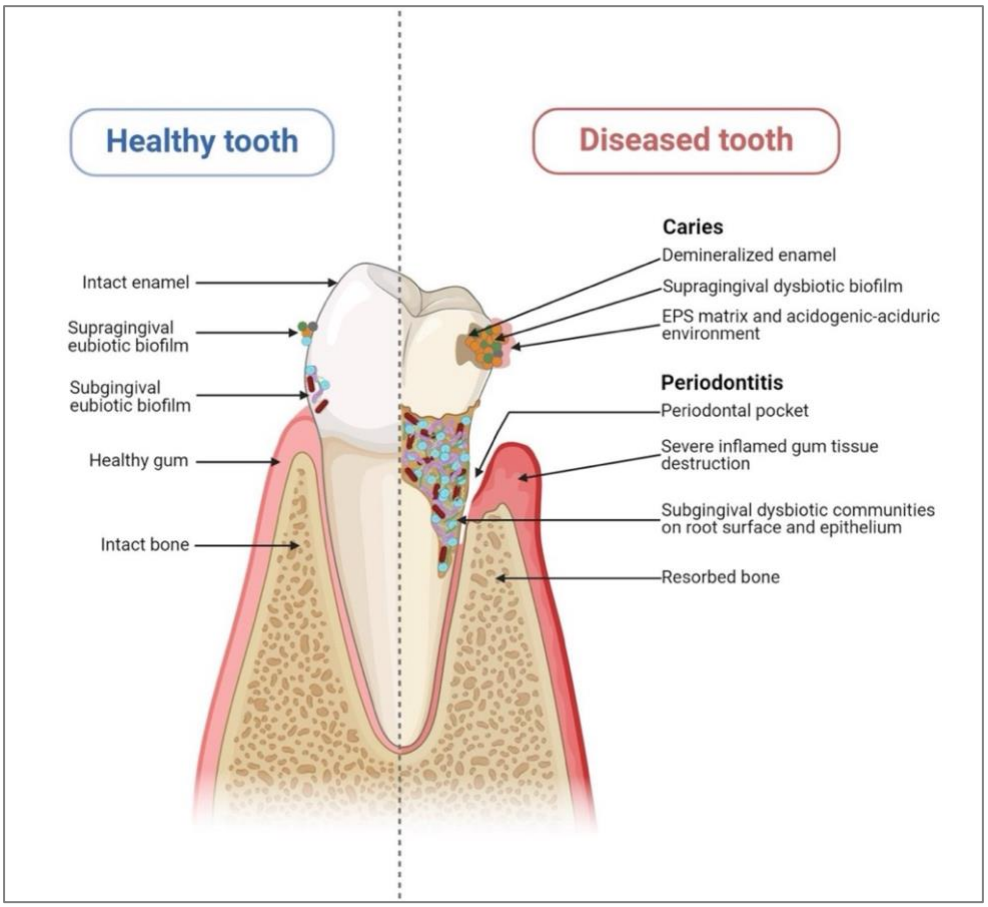


FIGURA 1. Dente saudável e dente com cárie dentária e periodontite. Representação esquemática das diferenças entre um dente saudável e um dente doente com cárie e periodontite. Fonte: Disponível em: https://biorender.com/. Acessado em: Agosto de 2026.

A doença cárie ainda afeta cerca de 50% das crianças brasileiras aos 5 anos e 95% da população adulta, segundo dados preliminares do SB Brasil (2020). Durante a gestação, alterações hormonais, mudanças alimentares, incluindo aumento do consumo de alimentos açucarados, modificações no ambiente bucal e o receio do tratamento odontológico podem aumentar a suscetibilidade à doença cárie (ORLOFF et al., 2016; CHO et al., 2020). Já a periodontite, nesse contexto, tem sido considerada um possível fator de risco para desfechos gestacionais adversos, como o parto prematuro e o baixo peso ao nascer (WU et al., 2015; NANNAN et al., 2022) (Figura 2).

Assim, a intervenção odontológica adequada durante a gestação pode beneficiar a saúde materna e infantil, sendo o segundo trimestre considerado um período favorável à realização de

tratamentos odontológicos eletivos, embora procedimentos de urgência possam ser realizados em qualquer fase gestacional quando clinicamente indicados (NANNAN et al., 2022).

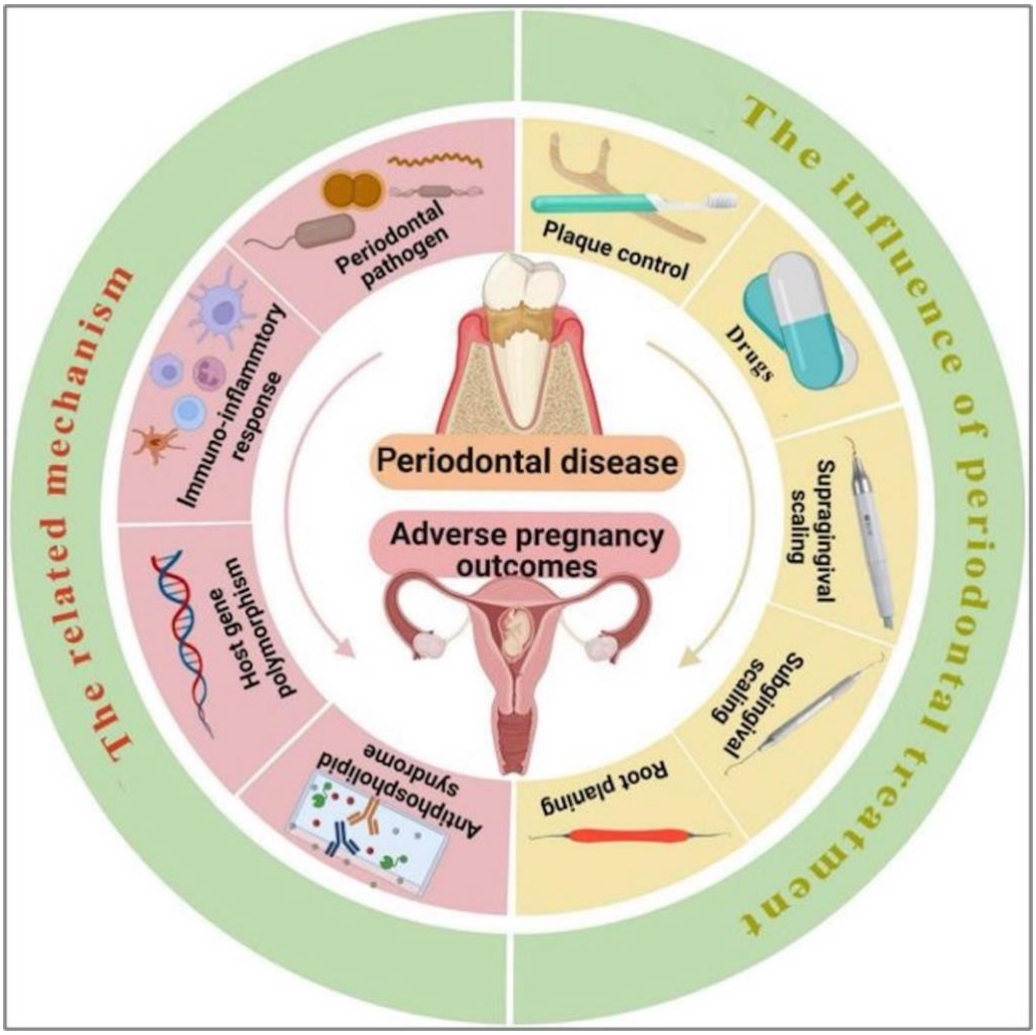


FIGURA 2. Doença periodontal e desfechos gestacionais adversos. O lado esquerdo da imagem ilustra os mecanismos associados à doença periodontal que levam a desfechos gestacionais adversos, incluindo a infecção por patógenos periodontais e alterações nos níveis de mediadores inflamatórios. O lado direito da imagem mostra o efeito do tratamento periodontal sobre esses desfechos gestacionais adversos; os métodos de intervenção incluem controle de placa, administração de medicamentos, raspagem supragengival e raspagem e alisamento radicular (subgengival). Fonte: Criado com https://biorender.com/ por NANNAN et al., 2022. Acessado em: Agosto de 2026.

Além da relevância clínica dessas doenças, o estudo do microbioma oral tornou-se central na pesquisa contemporânea. A microbiota oral corresponde ao conjunto de microrganismos presentes na cavidade bucal, enquanto o termo microbioma, em sentido mais amplo, compreende esses

microrganismos, seus genes e suas interações com o ambiente. Esse ecossistema inclui bactérias, fungos, vírus e protozoários que habitam a cavidade bucal humana (DEO et al., 2019). Como porta de entrada do organismo, a boca participa da primeira etapa do processo digestivo, permitindo que microrganismos e moléculas sejam deglutidos e possam interagir com outros sistemas orgânicos (SABELLA et al., 2021; RIZZARDI et al., 2022). Dessa forma, o ecossistema microbiano oral participa de interações relevantes não apenas para a saúde bucal, mas também para processos imunológicos e sistêmicos.

Estudos recentes têm demonstrado associações entre alterações da saúde bucal e condições sistêmicas, incluindo doenças cardiovasculares, disfunção cognitiva e demência (HARDING; SINGHRAO, 2022; TUGANBAEV; YOSHIDA; HONDA, 2022). Especialmente na infância, alterações no estabelecimento inicial das comunidades microbianas têm sido investigadas por sua possível relação com desfechos de saúde ao longo da vida, incluindo doenças autoimunes, alergias e câncer (DEO et al., 2019; WANG et al., 2026).

A composição do microbioma oral é influenciada por fatores ambientais e comportamentais, por isso escolhas relacionadas ao estilo de vida podem modificar e limitar a diversidade da microbiota, e nesse sentido a alimentação se destaca como um importante modulador da diversidade microbiana (ESBERG et al., 2020; OBA et al., 2020). Além disso, características parentais, como hábitos alimentares, higiene bucal e microbiota oral materna (Figura 3), podem influenciar o estabelecimento inicial do microbioma infantil (LUNDGREN et al., 2018; DASHPER et al., 2019). O equilíbrio das comunidades microbianas e de suas interações com o hospedeiro é fundamental para a manutenção da homeostase, enquanto alterações nesse equilíbrio podem favorecer estados de disbiose associados ao desenvolvimento de doenças.

Compreender quais fatores podem ser modificados para favorecer o estabelecimento e a manutenção de comunidades microbianas compatíveis com a saúde é essencial para estratégias de promoção de saúde, uma vez que a saúde bucal integra de forma indissociável a saúde geral. Nesse sentido, os avanços nas técnicas moleculares de identificação microbiana têm ampliado a compreensão sobre a composição, a diversidade e as mudanças que ocorrem nas comunidades bacterianas ao longo do tempo. Entre essas ferramentas, destaca-se o sequenciamento do gene 16S rRNA, que possibilita a caracterização de comunidades bacterianas complexas sem depender exclusivamente de métodos tradicionais de cultivo.

O sequenciamento do gene 16S rRNA é uma abordagem amplamente utilizada para caracterizar o componente bacteriano de comunidades microbianas presentes em diferentes ambientes, incluindo a cavidade bucal (HUMAN MICROBIOME PROJECT CONSORTIUM, 2012). O método baseia-se na análise do gene que codifica o RNA ribossômico 16S, presente em bactérias e que contém regiões conservadas intercaladas por regiões hipervariáveis. Enquanto as regiões conservadas permitem a amplificação do gene em diferentes grupos bacterianos, as regiões hipervariáveis apresentam diferenças em suas sequências que possibilitam a identificação e a classificação taxonômica dos microrganismos (PORETSKY et al., 2014). Por meio do sequenciamento dessas regiões e da posterior análise bioinformática, é possível determinar a composição e a diversidade das comunidades bacterianas, inclusive de microrganismos que não são facilmente cultiváveis por métodos convencionais. Dessa forma, o sequenciamento do gene 16S rRNA tem contribuído significativamente para a compreensão da estrutura e da dinâmica do microbioma oral, permitindo investigar alterações na sua composição associada à saúde, à doença e a diferentes fases do desenvolvimento humano. Sua aplicação em estudos longitudinais da díade mãe–bebê possibilita, ainda, acompanhar as mudanças que ocorrem durante o estabelecimento da microbiota bucal do bebê e investigar similaridades entre os perfis bacterianos maternos e infantis ao longo dos primeiros meses de vida (DZIDIC et al., 2018).



FIGURA 3. Desenvolvimento do microbioma oral na díade mãe–bebê. Representação esquemática do estabelecimento e desenvolvimento do microbioma oral na díade mãe–bebê, desde o terceiro trimestre gestacional até os primeiros meses de vida, destacando a continuidade temporal das interações materno-infantis e a dinâmica de colonização microbiana oral. Fonte: elaboração própria, criada com auxílio de ferramenta de ilustração digital. Data: Agosto de 2026.

Entretanto, a compreensão do ecossistema oral não se limita à caracterização de sua composição microbiana. As interações entre os microrganismos e o hospedeiro envolvem também diferentes componentes da resposta imunológica, entre os quais se destacam as citocinas. Citocinas pró e anti-inflamatórias presentes na saliva refletem a atividade imunológica local e têm sido investigadas como potenciais biomarcadores de processos inflamatórios bucais, incluindo a cárie dentária (LEME et al., 2022; DAVIDOVICH et al., 2024). Entretanto, ainda são escassos os estudos que avaliam simultaneamente o microbioma oral e o perfil inflamatório salivar na díade mãe–bebê durante os primeiros meses de vida.

Além dos componentes microbianos e imunológicos, a investigação das proteínas presentes na saliva oferece outra perspectiva para a compreensão do ecossistema oral. Nesse contexto, a proteômica salivar constitui uma abordagem complementar capaz de caracterizar conjuntos de proteínas e peptídeos e investigar suas possíveis relações com condições fisiológicas e patológicas. A saliva exibe uma ampla gama de proteínas e peptídeos bioativos com função na defesa e regeneração oral, moléculas tais como mucinas, lactoferrina e imunoglobulinas (VITORINO et al., 2004; PLETCHER; PIGNONE, 2011). Técnicas avançadas, como a espectrometria de massas, permitem identificar a presença ou ausência desses múltiplos biomarcadores, como uma potencial fonte de detecção precoce, monitoramento da progressão e/ou resposta ao tratamento de muitas doenças bucais e sistêmicas (BALDINI et al., 2011; WEI et al., 2013; ALQADERI et al., 2022), diferentes tipos de câncer (QIU et al., 2009; WU; WANG; ZHANG, 2009; LIN et al., 2010), bem como a doença periodontal (JAVAID et al., 2016, FORATORI-JUNIOR et al., 2026) e cárie dentária (GAO et al., 2016; AO et al., 2017; GOODSON et al., 2020; OLIVEIRA et al., 2022). A espectrometria de massas baseia-se na ionização das moléculas e na determinação da relação massa/carga (m/z) dos íons gerados, permitindo obter informações sobre sua composição molecular. Em abordagens proteômicas, as proteínas são geralmente digeridas em peptídeos, que são separados por cromatografia líquida e posteriormente analisados pelo espectrômetro de massas. Os espectros obtidos são então processados por ferramentas bioinformáticas e comparados a bancos de dados de sequências proteicas, possibilitando a identificação das proteínas presentes na amostra (ZHANG et al., 2025).

Dentro desse contexto, a coleta de amostras de saliva é um procedimento não invasivo, indolor e uma opção simples, segura e barata em comparação à coleta de outros componentes do corpo (PRODAN et al., 2016; FEY; BIKKER; HESSE, 2024). Particularmente em certos grupos

específicos, como crianças, bebês e lactentes, a saliva pode ser considerada como o fluído diagnóstico ideal, pois sua coleta reduz o desconforto e garante cooperação (PAPPA; KOUSVELARI; VASTARDIS, 2019). Embora a análise proteômica tenha sido amplamente explorada nos últimos anos, os dados sobre perfil proteômico salivar de gestantes e, particularmente, sobre as possíveis similaridades entre mães e seus bebês ainda são escassos (DEY et al., 2020; BALAN et al., 2021) sendo a investigação de mulheres gestantes obesas e com periodontite a mais comum (FORATORI-JUNIOR et al., 2023).

A identificação de biomarcadores moleculares e a caracterização integrada dos componentes microbianos, imunológicos e proteicos da saliva podem ampliar a compreensão dos processos biológicos envolvidos na saúde materno-infantil e, futuramente, contribuir para estratégias de avaliação de risco e prevenção. Considerando que a mãe constitui uma importante fonte de exposição microbiana para o bebê e compartilha com ele fatores genéticos, ambientais e comportamentais, investigar a díade mãe–bebê é fundamental para compreender os estágios iniciais de estabelecimento do ecossistema oral infantil (Figura 3).

Diante da escassez de evidências longitudinais integrando microbioma oral, citocinas salivares e proteômica na díade gestante/mãe–bebê, torna-se necessária uma abordagem que considere simultaneamente saúde bucal materna, fatores alimentares e biomarcadores salivares. Essa lacuna fundamenta a presente tese e motivou a investigação integrada do ecossistema oral materno-infantil ao longo da gestação e dos primeiros meses de vida.

2. OBJETIVOS

2.1 Objetivo Geral

Esta tese tem como objetivo investigar longitudinalmente a relação entre a saúde bucal, o microbioma salivar, o perfil proteômico e o perfil imunológico salivar de gestantes/mães e seus respectivos bebês durante os primeiros meses de vida.

2.2 Objetivos Específicos

1. Avaliar longitudinalmente o microbioma oral e o perfil de citocinas salivares de gestantes/mães e de seus respectivos bebês, desde o terceiro trimestre gestacional até 1, 3 e 6 meses pós-parto.

2. Caracterizar o perfil proteômico salivar da díade mãe–bebê, desde o terceiro trimestre gestacional até o primeiro mês de vida do lactente.

CAPÍTULO 1: Artigo aceito para publicação:

“Ecological succession links mother–infant oral microbiota colonization across the first six months of life”

A boca do bebê passa por grandes mudanças nos primeiros meses de vida. Acompanhamos 27 mães e seus bebês desde a gestação até os seis meses após o nascimento para entender como os microrganismos e componentes da resposta imunológica presentes na saliva se modificam nesse período. Observamos que a microbiota oral dos bebês se tornou progressivamente mais complexa e mais semelhante à das mães. Alguns grupos de bactérias relacionados à cárie já estavam presentes nos primeiros meses, enquanto aqueles associados às doenças periodontais diminuíram ao longo do tempo. Os resultados ajudam a compreender como o ambiente bucal se estabelece no início da vida e reforçam a importância dos cuidados com a saúde bucal da mãe durante a gestação e após o nascimento.

Este artigo foi aceito para publicação pela revista científica “*Journal of Dentistry*” - ISSN: 0300-5712. Fator de impacto (*JCR ou Scopus Citescore*): 7.8. *Highest percentile* (JCR ou Scopus): 93%. Qualis da revista de acordo com o percentil: conceito A1 na Medicina 1.

Ecological succession links mother–infant oral microbiota colonization across the first six months of life

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Availability of Data: The raw data of all libraries generated during this study are publicly available at the Sequence Read Archive (SRA) portal of NCBI under accession number BioProject: PRJNA1279152. SRA: https://www.ncbi.nlm.nih.gov/bioproject/ PRJNA1279152.

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ABSTRACT

Objective: This study evaluated salivary cytokine profiles and characterized longitudinal salivary microbiome dynamics in 27 mother-infant dyads, from third trimester of pregnancy through 1, 3, and 6 months postpartum. **Methods:** Mother-infant dyads were prospectively recruited according to predefined inclusion/exclusion criteria. Maternal oral health outcomes were assessed using standardized DMFT and CPI indices, supported by socioeconomic and dietary questionnaire. Saliva samples were subjected to multiplex bead–based assays for cytokine quantification and microbiome profiles were generated using 16S rRNA sequencing. Potential confounding factors were assessed due to their influence on early-life oral microbiome development. **Results:** Despite mid-level socioeconomic backgrounds mothers exhibited a high prevalence of dental caries (77.8%) and periodontal alterations (59.2%). Salivary maternal microbiome alpha-diversity remained temporal stable and significantly higher than that of infants ($p<0.05$). Infant microbiomes showed early variability and exhibited greater similarity to maternal profiles by six months (beta-diversity, $p<0.05$). Infant early communities were dominated by *Streptococcus*, followed by increases in *Prevotella*, *Veillonella*, and *Rothia*. Cariogenic genera persisted throughout infancy, whereas periodontal-associated taxa (*Aggregatibacter*, *Fusobacterium*) declined and were absent by six months. Distinct cytokine patterns were observed between mothers and infants, with consistent differences in IL-8 and IL-12 and time-dependent differences in IFN-γ, IL-10, IL-13, and IL-1β. **Conclusions:** These findings highlight a dynamic ecological succession of the infant salivary microbiome and are consistent with the concept of early-life microbial imprinting within the context of maternal–infant interactions and shared environmental exposures. The early persistence of cariogenic taxa reinforces the importance of maternal oral health interventions during this critical developmental window. **Clinical significance:** Early-life oral microbiome development is associated with maternal oral health and may reflect maternal–infant microbial sharing, with persistence of cariogenic taxa. This highlights the need for preventive dental care during pregnancy and early postpartum, in order to reduce infant risk and promote healthier oral microbiome trajectories.

Keywords: *infant-mother dyads; saliva; oral microbiome; microbial initial acquisition; cytokines.*

INTRODUCTION

Pregnancy and early infancy represent critical windows of physiological change and microbial community assembly, during which early exposures and ecological processes can shape long-term host health. The oral cavity harbors one of the most diverse microbial communities in the human body and functions as a major gateway to the gastrointestinal tract and systemic circulation. Oral microorganisms and microbial products are continuously delivered to downstream sites through swallowing and may disseminate beyond the oral ecosystem [1,2]. Accordingly, early-life microbial dynamics in the mouth should be viewed as part of a broader host-associated metacommunity in which dispersal, colonization, and selection occur across connected body sites.

The establishment of the infant oral microbiome is shaped by caregiver factors such as diet, hygiene practices, oral health status, and the composition of caregiver microbial communities. Lifestyle factors—particularly diet—can substantially modulate microbial diversity and functional potential [3,4] . Importantly, early-life microbial colonization trajectories have been linked to long-term health outcomes across the lifespan, including immune dysregulation, allergies, autoimmune diseases, and cancer susceptibility [5]. Understanding the ecological dynamics of early oral microbial assembly is therefore essential for preventive strategies with implications extending well beyond the oral cavity.

Oral biofilm-mediated diseases such as dental caries and periodontitis remain highly prevalent in the world population. Periodontal disease may contribute to adverse pregnancy outcomes, including preterm birth and low birth weight [6]. Maternal oral health influences both maternal quality of life and the microbial environment to which the infant is exposed [7]. Although maternal-to-infant microbial transmission is increasingly supported, transmission does not necessarily imply stable colonization, which depends on host–microbe interactions, ecological constraints, and habitat filtering [8]. These early microbial dynamics may influence both oral and systemic outcomes, including dental caries and obesity [9].

Emerging evidence further suggests that dental caries is driven by complex interactions between microbial ecology and host immunity. Studies have reported increased salivary levels of pro-inflammatory cytokines—including interleukin-1 (IL-1), interleukin-6 (IL-6), and tumor necrosis factor alpha (TNF-α)—in individuals with active dental caries, supporting a role for inflammatory pathways in lesion progression [10–12]. However, despite extensive research in

children, studies simultaneously evaluating salivary cytokine profiles and oral microbiome composition in very young infants and their mothers remain limited.

Given the scarcity of longitudinal evidence integrating maternal oral health, diet, microbial community structure, and inflammatory markers during the earliest stages of life, the primary research question of this study was whether maternal oral health status, maternal oral microbiome and salivary inflammatory profiles during late pregnancy are associated with the early establishment and temporal dynamics of the infant oral microbiome. We hypothesized that maternal oral microbial and immunological profiles would influence the initial microbial colonization patterns in infants.

Accordingly, this study aimed to characterize the salivary microbiome and cytokine profiles of women in the third trimester of pregnancy and to longitudinally assess maternal and infant oral microbiome development at 1, 3, and 6 months postpartum.

Understanding early-life oral microbial assembly provides a powerful framework to reveal how maternal and environmental factors drive microbiome development with potential lasting health consequences

MATERIAL AND METHODS

Ethics statement

Following the Declaration of Helsinki, this longitudinal observational study was performed between November 2023 and July 2024, after ethical approval (Ethics Committee of the University São Francisco - protocol number: 65319322.2.0000.5514) and obtaining written informed consent.

Study design and population

This study conveniently recruited 27 pregnant women (PW) and their infants from the largest Gynecology and Obstetrics Clinic of Extrema, Brazil. Extrema city is located in the south of Minas Gerais State, has ≅36.951 inhabitants, presents a human developmental index of 0.732, and optimally fluoridated tap water (0.69 mg F/L).

This study was part of a larger investigation involving 104 participants. PW in their third

trimester were eligible for inclusion if they completed one salivary sample collection during pregnancy and provided their infants' saliva samples at 1, 3, and 6 months of age.

Exclusion criteria were being in the first or second trimester of pregnancy, use of antibiotics, corticosteroids, or antihypertensive medications during the study period or within the month preceding enrollment, declining participation, presenting neuromotor or communication impairments or psychiatric disorders, or discontinuing participation after delivery (Figure 1). All enrolled infants were healthy, born at term, and had no congenital anomalies or chronic medical conditions at birth.

Of the participants enrolled in the larger study, 45 completed the baseline interview and saliva collection during pregnancy. However, only 27 mother–infant dyads completed all scheduled follow-up visits and sample collections through 6 months postpartum. Therefore, only complete mother–infant dyads were included in the present longitudinal analysis. Then, a total of 27 women and their infants met the criteria and were included in this follow-up study, yielding 54 participants. Individuals were classified into three groups: pregnant women (PW group), mothers (M group – the same participant evaluated after delivery), and infants (IF group).



Figure 1. Flow chart of the sample selection.

Assessment of pregnant women's oral health, dietary practices and post-delivery information

The oral examination was conducted in the clinic waiting room. After that, PW had their teeth cleaned and dried with gauze (Medi House, São Paulo-SP, Brazil), and the diagnosis of dental caries (Decayed, Missing, and Filled Surfaces/Teeth - DMFS/DMFT) and periodontal evaluation (Community Periodontal Index (CPI) was performed according to the World Health Organization (WHO) criteria[13,14]. For analytical purposes, periodontal status was dichotomized: sextants with an absence of gingival bleeding, calculus, and periodontal pockets were classified as healthy (CPI = 0), while those presenting at least one of these clinical signs were classified as unhealthy (CPI ≥ 1). The diagnosis was completed under headset light (Sfl5540, Wat Nichia Philips, Brazil), with a clinical mirror and a ball-ended dental probe (Indusbello, Londrina- PR, Brazil). Clinical examination was performed by one dentist, previously calibrated by a gold standard examiner, who received all the theoretical and practical instructions regarding the criteria to be used. About 10% of the sample was re-examined during the calibration process with a 1-week interval. A Kappa statistic was calculated to guarantee a homogeneous intra-examiner assessment, indicating excellent agreement, with a value of 0.86. Pregnant women who needed dental treatment were referred to clinical therapy.

Baseline interviews were conducted during the third trimester of pregnancy to collect maternal demographic, socioeconomic, dietary, and oral health information. Dietary practices during pregnancy were measured using a structured and simplified questionnaire recommended by the WHO to verify the frequency of consumption of certain food groups by adults. Questions about to socioeconomic status, including family income and educational attainment, were also added to the questionnaire, as follows: “What is your family income?” and “What is your education level?”. Additionally, the age and initial and final weights of the pregnant women were collected through medical record, and the body mass index (BMI) was calculated according to the WHO [15,16]. Post-delivery, information regarding gestational age at delivery, delivery mode, infant sex, birth weight and length (obtained from the infant health record) and type of feeding (exclusive breastfeeding, formula feeding, or mixed feeding), was collected during the first follow-up visit, when the infant was one month old. Information regarding infant antibiotic exposure was collected throughout the follow-up period, and none of the infants were exposed to antibiotics during the study.

Saliva samples collection

Saliva from the PW was collected during the dental examination. Postpartum women were asked to refrain from eating or brushing their teeth for 1 hour before saliva collection, which was usually also performed in the morning. They were also instructed to refrain from feeding their babies. All the saliva samples were collected according to the Manual of Procedures for Human Microbiome Project[17], with minor modifications, as described below.

At least 500 microliters of unstimulated mothers' saliva was collected by passive drooling for about 3 minutes in plastic cups and transferred to microcentrifuge tubes using sterilized disposable syringes. Baby saliva was collected using SalivaBio's Infant Swab (SIS) Method (Salimetrics, USA) and the Salivette device (Sarstedt, Germany), which provide a clean and less invasive collection approach. The procedure involved two steps: 1. The roll was inserted into the baby's mouth until it was soaked with saliva; 2. The roll was removed and inserted into the Salivette plastic tube. Saliva was kept refrigerated during the entire collection period. All saliva samples were transported under refrigeration to the Laboratory of Clinical and Molecular Microbiology. The samples obtained with Salivette were centrifuged at 5,500 rpm for 10 minutes under refrigeration, and then the supernatant was transferred to 1.5 ml microtubes. All samples were frozen at -80°C [18].

DNA Extraction, Library Preparation and 16S rRNA Sequencing

DNA extraction from saliva samples was conducted using the QIAmp® DNA Mini Kit, following the provided instructions and guidelines from Qiagen (Isolation of DNA from saliva; (QA19s Jul-10) (Hilden, Germany). Subsequent DNA quantification was performed using SpectraMax iD3 Microplate Reader (Molecular Devices, San Jose, USA).

Barcoding and library preparation were performed using the Illumina 16S Metagenomic Sequencing Library Preparation Kit (Illumina®, San Diego, CA, USA), following the manufacturer's instructions. The V3–V4 hypervariable region of the bacterial 16S rRNA gene was amplified using 341F and 805R primers attached with the Nextera adapters as defined by the kit. Libraries were indexed, pooled, and sequenced on the Illumina NextSeq 2000 platform (paired-end, 2 × 300 bp) [19–21]. Additional details regarding primer sequences, PCR conditions, and library preparation are provided in Supplementary Materials.

A total of 189 saliva samples were collected according to the study protocol, of which 173 yielded sufficient DNA for bacterial 16S rRNA gene sequencing and subsequent data analyses.

Multiplex cytokine analysis

Saliva samples collected from participants were analyzed using a multiplex cytokine bead–based assay, following the technical guidelines supplied by the kit manufacturer. The measurements were performed with the Luminex Performance Assay (R&D Systems, Minneapolis, MN, USA). All reagents, standards, and biological samples were prepared exactly according to the provided instructions.

For each reaction, 50 µl of either the standard or the saliva sample was dispensed into the designated wells. The premixed microparticle suspension was gently vortexed for approximately 20 seconds, after which 50 µl of this bead mixture was added to each well. The plate was then sealed and placed on an orbital microplate shaker at 800 rpm for a 2-hour incubation at room temperature. Washing steps were carried out using a magnetic plate holder. After positioning the plate on the magnet for one minute, the liquid was removed, each well was filled with 100 µl of Wash Buffer, and the plate was left for another minute before aspirating again. This procedure was repeated three times. Subsequently, 50 µl of the diluted biotin-antibody mixture was added to each well. The plate was covered with a foil seal and incubated for 1 hour under the same shaking conditions. After another wash cycle, 50 µl of streptavidin-PE was added, followed by a 30-minute incubation. The wells were washed once more, and the beads were finally resuspended in 100 µl of wash buffer and mixed for 2 minutes at 800 rpm.

Data acquisition was performed using the Luminex 200 Milliplex System (Merck, Darmstadt, Germany), and cytokine concentrations were processed and interpreted using the Milliplex Analyst software. The cytokines examined in this study included IFN-γ, IL-1β, IL-6, IL-8, IL-10, IL-12, IL-13, and TNF-α [22].

Bioinformatics and Statical Analyses

The 16S rRNA amplicon sequencing data were processed using QIIME 2 (version 2024.10.1) along with Greengenes 16S rRNA database (release 2022.10) for taxonomic classification [21] as

well as analyses of alpha and beta diversity and relative abundance. Briefly, paired reads were trimmed using cutadapt to remove amplicon primers; further trimmed with a length of 250 before the filtering, denoising and merge; and further filtered to retain a feature shown in at least two samples using DADA2. This preprocessing resulted in an average of 451,900 reads and 6,659 feature frequencies per sample with a length of around 460 bp (Supplementary Materials). Taxonomy was subsequently assigned with feature-classifier using V3-V4 references trained from the Greengenes 16S rRNA database. All the samples were included in the study without any dropouts or missing data, due to the relatively small sample size.

Statistical analyses were performed using GraphPad Prism version 10.0.0 (GraphPad Software, Boston, Massachusetts USA) with a level of significance <5%. Friedman and Mann Whitney test were used for alpha diversity and relative abundance and cytokines levels evaluations. Friedman was used for the whitin-group, repeated-measures comparisons across time (PW→M1→M3→M6 and B1→B3→B6) and Mann-Whitney test for the cross-sectional mother x (versus) infant comparisons at each timepoint (M1 x B1, M3 x B3, M6 x B6). When suitable, post hoc tests with Bonferroni correction were applied. PERMANOVA was applied for beta diversity analyses and ANCOM-BC for genus/family increase or decrease ($p < 0.05$).

RESULTS

Populations' characteristics

We included 27 mother–infant dyads in our study cohort, which were followed longitudinally at four time points. Saliva samples were collected from mothers during the third trimester of gestation and at 1, 3, and 6 months postpartum, while infant samples were collected at 1, 3, and 6 months postpartum.

The mothers' ages ranged from 17 to 53 years, with a mean age ± standard deviation (SD) of 28.5 ± 7.4 years. The mean pre-pregnancy BMI was 25.08 kg/m², and the mean BMI in the third trimester was 28.35 kg/m². All infants were born full term; 17 were male and 10 were female. The mean infant's birth weight was 3.095 kg, and mean infant's length was 48 cm. The majority of deliveries were performed by cesarean section (92.6%), whereas only 7.4% occurred via the vaginal route. None of the infants were exposed to antibiotics during the study period. Regarding dietary practices, 65.4% of pregnant women reported consuming fruits and only 34,6% consume

them daily. Fifty percent of the participants included vegetables in their routine diet. Considering sucrose consumption in the solid and in the liquid form, 4% and 15% reported daily consumption and 26% and 33% a week consumption, respectively. Soft drinks or lemonade and tea or coffee with sugar were mostly consumed monthly. Regarding periodontal status (healthy: CPI = 0; unhealthy: CPI ≥ 1), 59.2% of the pregnant women presented at least one unhealthy sextant. The patients' characteristics are displayed in Table 1.

Table 1. Characteristics of the study cohort.

	Participants	
	n	%
	Infants	
Milk fed at 1 month		
Breast milk	19	70.4
Formula milk	4	14.8
Breast + formula milk	4	14.8
	Pregnant Women/Mothers	
Periodontal Index (CPI)		
All sextants healthy	11	40.8
At least 1 sextant unhealthy	9	33.3
More than 2 sextants unhealthy	7	25.9
Dental Caries index (DMFT)		
0	6	22.2
1-8	13	48.1
≥8	8	29.7
Income (USD/month)		
<500	16	59.3
500 to 1000	6	22.2
>1000	5	18.5
Education Status		
Middle school	3	11.1
High school	15	55.6
College	9	33.3

Dietary Practices	Consumption more than once a month n (%)	Consumption more than once a week n (%)	Consumption daily n (%)
Solid sucrose	19 (70)	7 (26)	1 (4)
Liquid sucrose	14 (52)	9 (33)	4 (15)
Soft drinks or lemonade	11 (41)	13 (48)	3 (11)
Tea with sugar	18 (67)	4 (15)	5 (18)
Coffee with sugar	12 (44)	11 (41)	4 (15)

BMI: Body Mass Index; DMFT: decayed missing or filled teeth (permanent); CPI: Community Periodontal Index.

Changes in the Salivary Microbiome with Age and Temporal Stability of the Maternal Oral Microbiome

Salivary microbiome diversity, as measured by the Shannon index, was temporal stable in pregnant women and mothers ($p>0.05$). In contrast, infants exhibited significantly lower alpha diversity values, with statistical difference between 1 month *versus* 6 months, as well as 3 months *versus* 6 months ($p<0.001$).

Across the mother-infant dyad, alpha diversity values were consistently and significantly higher in mothers compared to infants ($p<0.001$) (Figure 2). Infants displayed markedly lower initial alpha-diversity values, demonstrating the smallest variability at 1 month of age, followed by a progressive increase in both diversity and inter-individual variability across subsequent time points.

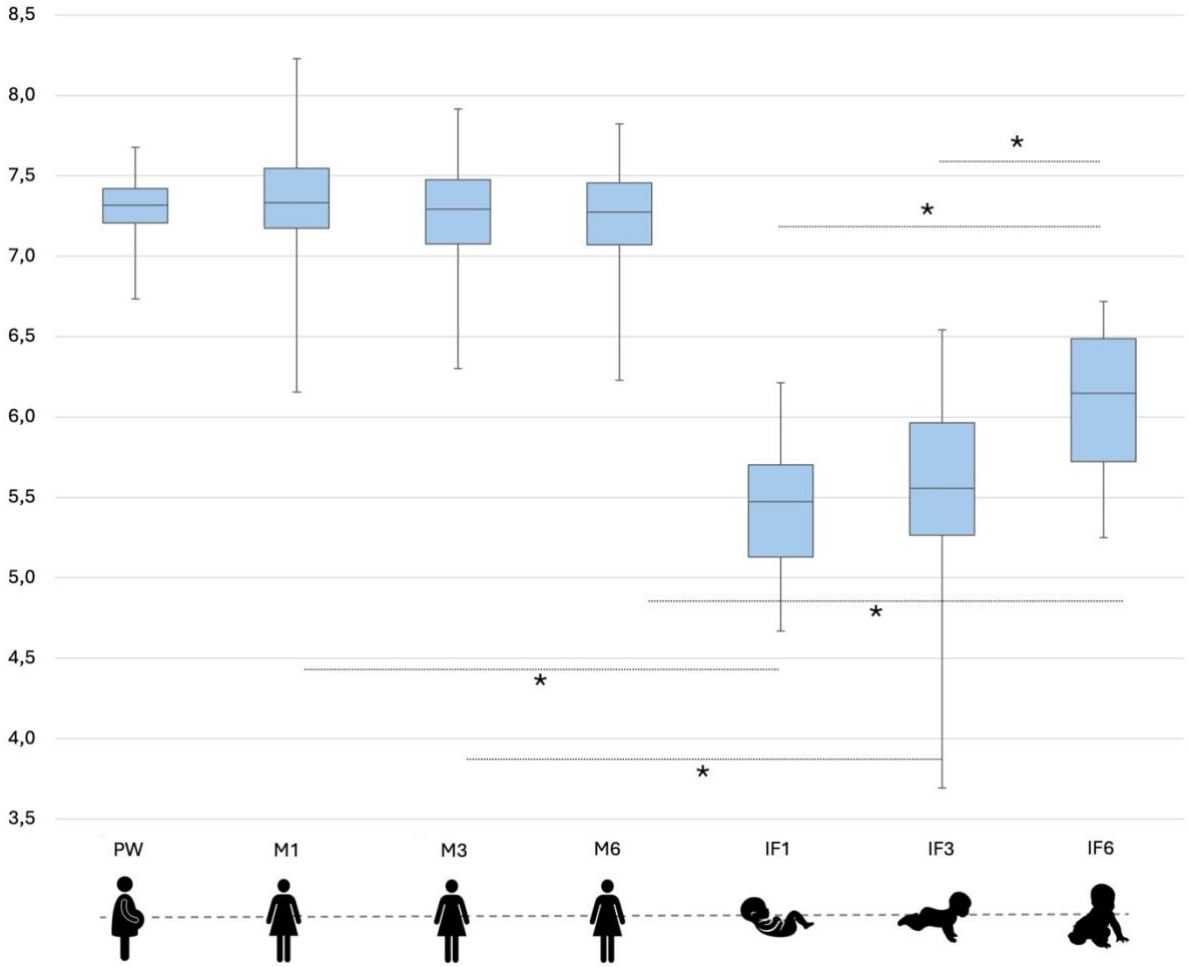


Figure 2. Alpha-diversity (Shannon Index) of the oral microbiome in each group. Boxplots show the Shannon diversity index of each group.

Temporal Dynamics and Differences in the Maternal and Infant Salivary Microbiome

The Principal Coordinates Analysis (PCoA) based on beta diversity metrics demonstrated significant differences in the salivary microbiota composition between maternal and infant samples at 1, 3, and 6 months of age, as confirmed by PERMANOVA (P=0.001 for all comparisons; Figure 3A–C).

Although significant differences between maternal and infant salivary microbiota persisted at 6 months (PERMANOVA, P=0.001; Figure 3C), the ordination plot suggests a visual tendency toward closer proximity between the groups compared with earlier time points. This observation should be interpreted cautiously, as no formal within-pair distance or dispersion analyses were performed to quantify temporal convergence. When all samples were analyzed together (Figure 3D), maternal and infant profiles remained significantly distinct (PERMANOVA, P=0.001), despite partial overlap at later time points.

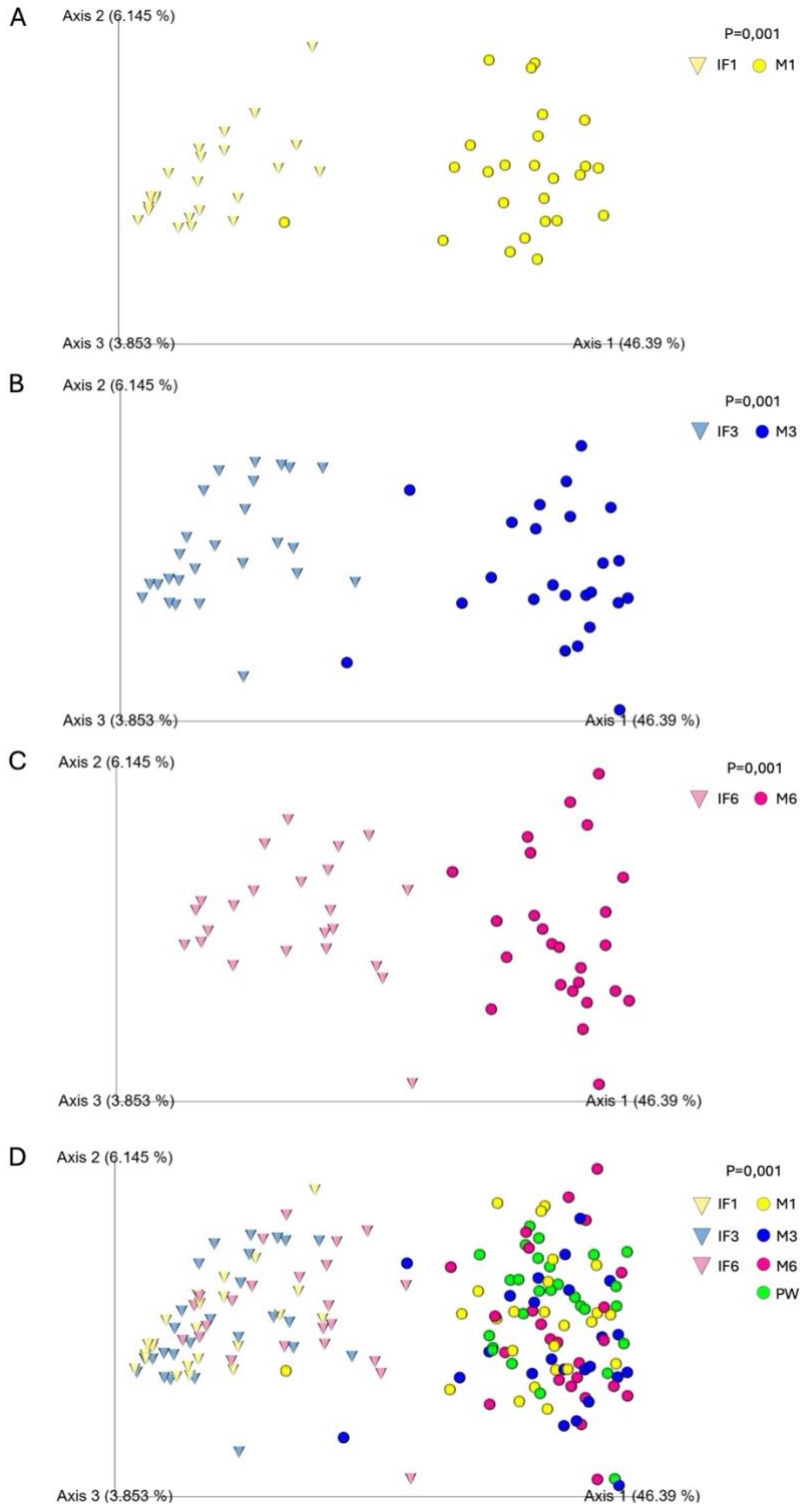


Figure 3. Comparison of the salivary microbiome of the infants and their mothers. Bray-Curtis Principal Coordinates Analysis (PCoA). The PCoA plots represent beta diversity distances between maternal and infant salivary microbiota samples at 1 month (A), 3 months (B), and 6 months postpartum (C), as well as all time points combined (D). Each point represents an individual sample, and the proximity between points reflects microbial compositional similarity based on Bray-Curtis dissimilarity.

Analysis of Relative Abundance of Salivary Microbiota

Analysis of the relative abundance of salivary microbiota revealed distinct compositional patterns between pregnant women, mothers, and their infants over time (Figure 4). The maternal microbiota, including from the third trimester through six months postpartum, exhibited a consistent and diverse community structure dominated by the following genera: *Streptococcus*, *Prevotella*, *Veillonella*, *Haemophilus*, *Leptotrichia*, *Neisseria*, *Rothia* and *Fusobacterium*. The only statistical difference found regarding these bacteria was in *Rothia* abundance, more specifically between PW and M1 ($p<0.05$ – Table 2).

In contrast, samples from infants showed a marked shift toward a low-diversity salivary microbiota compared to their mothers, initially dominated by *Streptococcus* from the first month of life. By six months, a gradual increase in diversity was observed, accompanied by a relative decrease in *Streptococcus* abundance and a progressive emergence of genera typically found in the maternal microbiota (Figure 4). The decrease in the *Streptococcus* genus was statistically significant regarding IF1 and IF6, as well as IF3 and IF6 ($p<0.05$ – Table 2). Significant increases were shown concerning *Prevotella*, *Veillonella*, and *Rothia* between IF1 and IF6 ($p<0.05$ – Table 2).

The relative genus abundance between mother-infant dyads across the evaluated periods of time revealed a statistical difference in 1. IF1 *versus* M1 - *Streptococcus*, *Prevotella*, *Veillonella*, *Haemophilus*, *Leptotrichia*, *Neisseria*, *Rothia and Fusobacterium* ($p<0.05$); 2. IF3 *versus* M3 - *Streptococcus*, *Prevotella*, *Haemophilus*, *Leptotrichia*, *Neisseria and Fusobacterium*; 3. IF6 *versus* M6: *Streptococcus*, *Prevotella*, *Haemophilus*, *Leptotrichia*, *Neisseria*, *Rothia and Fusobacterium.*

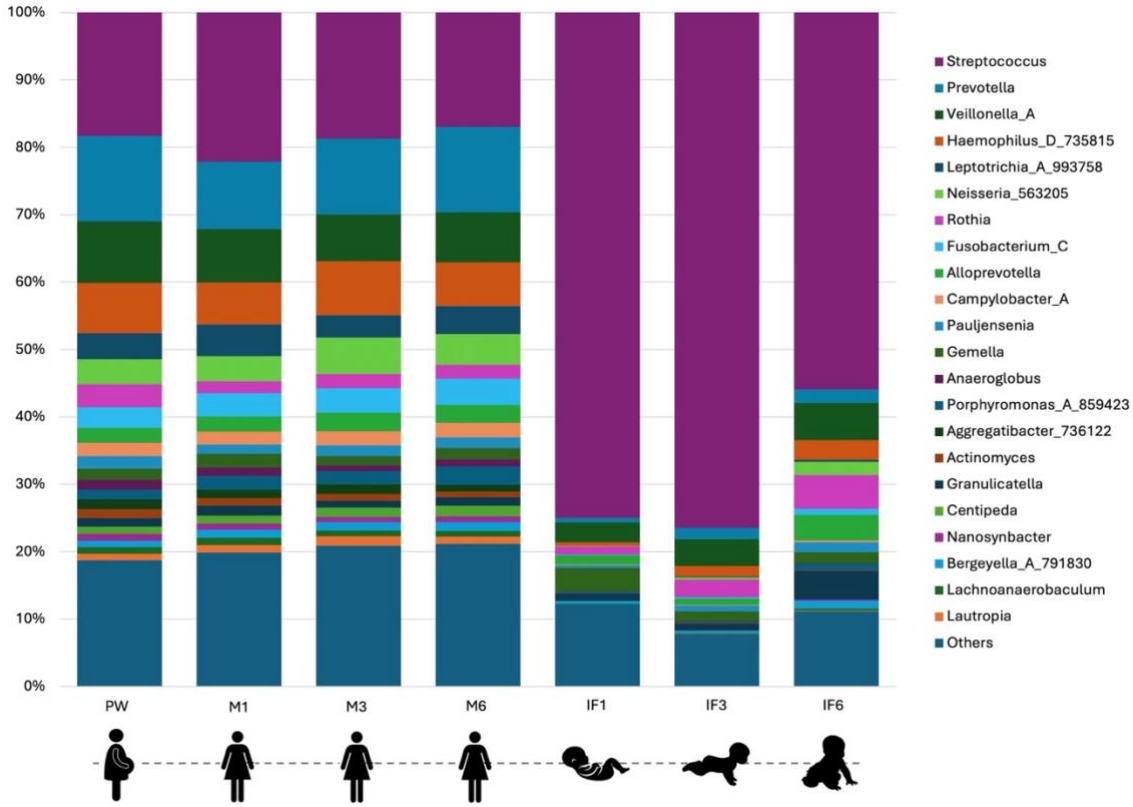


Figure 4. Analysis of the relative abundance of the salivary microbiome of the pregnant women, mothers and infants at different times. The genera shown are those with an abundance greater than 1%, sorted from bottom to top based on increasing average relative abundance according to the count number.

Table 2. Median and interquartile deviation of the most prevalent bacteria genera with statistically significant differences between the groups pregnant women (PW), mothers (M), and infants (IF) in 1, 3 and 6 months after birth.

Bacteria Genera	PW	M1	M3	M6	IF1	IF3	IF6
Streptococcus	18.83 (10.15) [a]	19.44 (9.07) [a]	16.08 (7.87) [a]	16.16 (8.84) [a]	72.93 (29.97) [a]	69.45 (18.45) [a]	51.04 (21.11) [b]
Prevotella	13.03 (5.28) [a]	10.60 (4.42) [a]	10.80 (5.74) [a]	11.50 (8.42) [a]	0 (0.33) [a]	0.37 (2.59) [ab]	1.77 (2.34) [b]
Veilonella	8.65 (3.74) [a]	7.29 (3.39) [a]	6.68 (3.71) [a]	5.84 (6.33) [a]	0.81 (1.89) [a]	4.29 (7.62) [ab]	3.58 (5.78) [b]
Haemophilus	6.71(4.2) [a]	5.25 (5.21) [a]	7.25 (5.07) [a]	5.82 (3.9) [a]	0 (0) [a]	0 (1.61) [a]	1.22 (3.38) [a]
Leptotrichia	3.66 (2.16) [a]	4.22 (3.21) [a]	2.82 (2.18) [a]	3.87 (2.55) [a]	0 (0) [a]	0 (0) [a]	0 (0) [a]
Neisseria	2.04 (1.85) [a]	3.37 (2.34) [a]	4.41 (3.15) [a]	4.09 (3.68) [a]	0 (0) [a]	0 (0) [a]	0 (3.02) [a]
Rothia	2.63 (1.88) [a]	1.67 (1.52) [b]	2.25 (1.4) [ab]	1.75 (1.01) [ab]	0.89 (1.72) [a]	2.27 (2.55) [ab]	4.70 (4.29) [b]
Fusobacterium	2.91 (2.14) [a]	3.24 (1.45) [a]	3.54 (2.55) [a]	3.78 (3.15) [a]	0 (0) [a]	0 (0.11) [a]	1.06 (1.18) [a]

Distinct lower case letters (a and b) indicate statistical differences among PW, M1, M3 and M6; and among IF1, IF 3, and IF 6 by the Friedman test (alpha<0.05). The Bonferroni correction was applied to prevent false positives.

Changes in the salivary microbiota across the mother-infant dyads

To investigate changes in the salivary microbiota across the mother-infant dyads, a differential abundance analysis was performed using 16S rRNA gene sequencing data. The resulting log fold change (LFC) bar plots display microbial taxa (at the genus level) that were significantly enriched or depleted in each group ($p<0.05$), enhancing the similarity the longer the follow-up period. The analysis was conducted using ANCOM-BC, a compositional statistical method designed to identify differentially abundant taxa while controlling for false discovery. In infants at one, three, and six months, the number of bacterial genera/families that were significantly increased and decreased in relation to the mother (reference) were respectively: 18 and 15; 15 and 9, and 2 and 29. The two genera that remained decreased after 6 months were *Centipeda* and *Leptotrichia* ($p<0.05$). Interestingly, classical cariogenic bacterial genera such as *Streptococcus* and *Lactobacillus* were enriched in all infant timepoints, suggesting their involvement in early colonization stages. Two genera closely related to periodontal disease, *Aggregatibacter* and *Fusobacterium*, are diminished in the first two time points and completely disappear in the last one: 6 months (Figure 5).

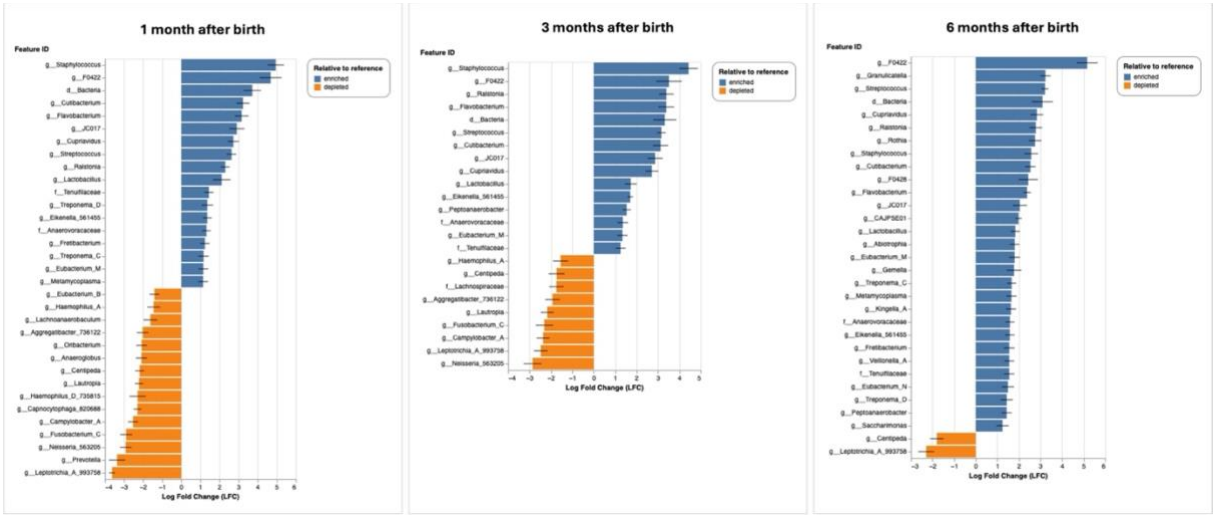


Figure 5. Differentially abundant oral microorganisms between mothers and infants during early life at months 1, 3, and 6. The genera with significant differences between the mothers and the infants were detected by log fold change (LFC) bar plots conducted using ANCOM-BC statistics.

Cytokine dynamics within the mother–infant dyads

Statistically significant differences between mothers and infants were identified at all three time points (1, 3, and 6 months) for IL-8 and IL-12 ($p < 0.05$). Significant differences within the dyads were detected only at 1 month for IFN-γ and IL-10 ($p < 0.05$), whereas IL-13 showed differences at 1 and 3 months ($p < 0.05$), and IL-1β at 3 and 6 months ($p < 0.05$). No significant differences in salivary concentrations of IL-6 and TNF-α were observed between mothers and infants (Table 3). No statistically significant differences were observed between all PW, M and IF.

Table 3. Median and interquartile deviation of the cytokines (pg/ml) regarding the groups pregnant women (PW), mothers (M), and infants (IF) at 1, 3, and 6 months after birth.

Cytokines	PW	M1	M3	M6	IF1	IF3	IF6
IFNγ	148.89 (231.60)	136.34 (271.52)*	111.72 (301.36)#	65.41 (114.07)	2104 (1862.14)*	638.56 (1363.99)#	207.23 (1206.72)
IL-1β	438.44 (816.83)	217.92 (695.08)	621.34 (607.71)#	142.7 (1639.71)$	307.46 (402.89)	111.365 (264.72)#	11.94 (207.20) $
IL-6	131.64 (130.83)	122.28 (341.61)	120.41 (326.46)	118.55 (216.53)	420.90 (361.26)	86.61 (116.26)	78.94 (266.07)
IL-8	9679 (17641)	7331 (8137.50)*	9873 (9490.50)#	6294 (9594.5)$	1302.5 (2350.36)*	645.21 (661.73)#	2018 (1904.22)$
IL-10	25.56 (22.50)	41.12 (25.45)*	27.3 (21.05)	37.32 (24,99)	356.36 (412.26)*	143.98 (225.93)	28.23 (158.64)
IL-12(p70)	13.26 (2.80)	13.26 (2,94)*	13.26 (4.475)#	13.26 (0)$	237.74 (299.95)*	110.32 (157.04)#	19.70 (147.6)$
IL-13	11.53 (0)	11.53 (0)*	11.53 (0)#	11.53 (0)	77.56 (260.00)*	81.14 (170.98)#	11.53 (131.94)
TNF- α	18.16 (6.47)	18.16 (5.76)	18.16 (18.35)	18.16 (2.33)	19.93 (18.46)	18.16 (5.40)	18.16 (3.30)

Identical symbols (*,* and #,# and $, $) indicate statistical differences between M1xIF1 (*), M3xIF3 (#), and M6xIF6 ($) by the Mann-Whitney test (alpha<0.05). Statistical differences by the Friedman test (alpha<0.05) among PW, M1, M3 and M6 were not found regarding all evaluated cytokines; and among IF1, IF2 and IF3 statistical differences were found only for IL-1B concerning IF1xIF6. The Bonferroni correction was applied to prevent false positives.

DISCUSSION

This longitudinal investigation characterized the early colonization and diversity of the oral microbiome in 27 healthy infants at 1, 3, and 6 months of age, alongside the salivary microbiomes of their mothers starting from the third trimester of pregnancy. Our goal was to characterize the initial microbial acquisition in infancy and assess the maternal association with oral microbiome development during this critical early-life window/period. Our findings suggest dynamic microbial maturation in early infancy and highlight a consistent maternal salivary microbiome profile from

pregnancy through the postpartum period.

The distinct alpha- and beta-diversity patterns observed between mothers and infants suggest that microbial maturation during early life follows a trajectory different from that of the relatively stable maternal salivary microbiome. The maternal salivary microbiome exhibited a consistent and diverse community structure throughout the study period, from the third trimester of pregnancy to the sixth month postpartum, with minimal fluctuations. In contrast, the infant salivary microbiome underwent marked temporal changes, characterized by a progressive increase in alpha diversity as the infants aged. These findings align with previous studies, which have demonstrated that microbial richness and diversity in the infant oral cavity increase over time through a dynamic ecological succession [8,23–25]. Likewise, beta diversity analyses also revealed significant differences between maternal and infant salivary microbiota at each time point, and the ordination plots suggested a tendency toward greater similarity between microbial profiles over time (Figure 2). Remarkably, by six months of age, the infant's salivary microbiome appeared visually more similar to that of the mother. Although this observation should be interpreted cautiously, it may reflect the cumulative effects of sustained maternal–infant contact and shared environmental exposures, which have been proposed as potential contributors to microbial exchange and ecological adaptation. Similar findings were reported by Li et al. [26], who also described an initial divergence at the postpartum period followed by greater similarity between maternal and infant microbiomes at 9 and 15 months. Additionally, previous studies have shown that this progressive ecological maturation leads the infant salivary microbiota to become increasingly complex and adult-like with age [8,25].

The temporal changes in the relative abundance of bacterial genera further supported the maturation of the infant salivary microbiome. At the genus level, mothers consistently presented a richer and more diverse microbial salivary community compared to their infants, with a greater number of genera shared between mothers and infants by six months of age. Across all time points, the core maternal salivary microbiome profile was predominantly composed of *Streptococcus*, *Prevotella*, *Veillonella*, *Haemophilus*, *Leptotrichia*, *Neisseria*, *Rothia* and *Fusobacterium*. Notably, clinical examination during the third trimester revealed that approximately 60% of pregnant participants presented with at least one unhealthy gingival sextant. This is particularly relevant given that increased abundance of genera such as *Veillonella*, *Prevotella and Fusobacterium* has been reported in pregnant women with gingivitis [27]. This is in line with the

conclusions of a systematic review, supporting that the oral microbial composition during pregnancy may be influenced by both local oral health status, and broader systemic conditions [7].

Conversely, bacteria strongly associated with periodontal disease, such as *Aggregatibacter* and *Fusobacterium*, were significantly depleted in infants compared with their mothers at 1 and 3 months of age and were no longer detectable at 6 months. This depletion is consistent with the fact that these taxa are commonly associated with periodontal dysbiosis in adults, a condition that would not be expected in healthy toothless infants. These observations are consistent with the findings of Reis et al. (2023) [28]. In their study comparing 50 children of parents with periodontitis to 50 children of periodontally healthy parents across different stages of dentition, they reported an association between parental periodontal status and the early oral microbiome composition of their children. Specifically, children of parents with periodontitis exhibited a progressively more pathogen-rich oral microbiome. However, Reis and colleagues identified the mixed dentition as the most critical phase for the establishment of periodontopathogenic species, a pattern that does not apply to the pre-dentition phase of the infants in our study. This is consistent with the depletion of pathogens at 1 and 3 months and their disappearance at 6 months, and this pattern may be explained by the absence of adequate ecological conditions—such as diet, oral hygiene, and, above all, the presence of teeth, the main substrate for the colonization of these microorganisms.

A notable observation was that the high prevalence of dental caries among pregnant participants may be relevant to the observed microbial salivary composition. Given the previous systematic review highlighting a scarcity of literature on maternal diet and the oral microbiome in mothers and babies [29], our study adds longitudinal data to help address this critical gap. Clinical assessments indicated that over 77.8% of the women had a DMFT index of at least 1, with restored teeth being the most frequent component. This finding may be associated with the consistent predominance of the genus *Streptococcus* in the maternal salivary microbiome, as this genus is well-established in the etiology of dental caries and is often associated with restored dentition [7]. Additionally, 30% of the pregnant women reported consuming sucrose-rich solid foods once a week or daily, and 48% reported similar consumption patterns for sucrose-rich beverages. Given that a diet high in fermentable carbohydrates is a well-established risk factor for the development of dental caries [30] this pattern may also have contributed to both the saliva composition and caries prevalence in this population. It is also important to consider the characteristics of the typical Brazilian diet, which is marked by a high intake of carbohydrates, substantial fruit consumption,

and relatively low protein content [31]. Interestingly, 16s rRNA sequencing studies have shown that mothers with dental caries may exert a stronger influence on their children's oral biofilm microbiota than caries-free mothers [23]. Because that study evaluated dental biofilm rather than saliva, direct comparisons with our findings should be made cautiously. Nevertheless, our results raise the possibility that similar mother–infant microbial associations may also be observed in the salivary microbiome.

The intricate processes governing how infants first acquire and later establish their oral microbial communities are still not fully understood. Our longitudinal results are consistent with the hypothesis of an early, *Streptococcus*-dominated phase followed by microbial diversification and maturation toward a more adult-like composition. Remarkably, a species of interest in the genus is *Streptococcus mutans,* of prime importance in the cariogenic process. A relatively low prevalence of *S. mutans* in such young babies has been previously reported [26]. In this regard, many investigations indicated that the onset of *S. mutans* colonization in the oral cavity may occur between 7 and 36 months of age. This delayed colonization window often coincides with primary tooth eruption and the introduction of complementary feeding, both of which fundamentally alter the oral ecological landscape and substantially influence microbial shifts [32–34]. Our data thus provide critical temporal insights into the pre-eruptive, *Streptococcus*-rich stage, preceding the establishment of a more complex cariogenic biofilm.

A significant demographic characteristic of our cohort was the high rate of cesarean sections, with nearly 25 out of 27 infants delivered via this route. While the mode of delivery has been associated with initial microbial acquisition, studies focusing specifically on the infant oral microbiome have demonstrated that these early differences are often transient and diminish rapidly over time [35].

Infant feeding practices represent another critical factor influencing salivary microbiota development. In our study, 70% were exclusively breastfed up to 6 months of age. This is consistent with a cross-sectional investigation reporting that the oral microbiota of 3-month-old infants differed markedly between those who were breastfed and those who received formula [36]. More recently, Li et al. (2023) [26] observed variations in microbial diversity and community composition between breastfed and non-breastfed infants, including a higher relative abundance of *Streptococcus* spp. and *Veillonella* spp. in breastfed infants and increased levels of *Rothia* spp.

among non-breastfed infants. Although these differences were not statistically significant in their cohort, their observations are broadly consistent with the microbial profile identified in our predominantly breastfed cohort. Interestingly, it has been demonstrated that children who are breastfed are at lower risk of early childhood caries than children who are bottle-fed [37]. Although breastfeeding may contribute to differences in microbial colonization, the high prevalence of exclusive breastfeeding in our cohort prevented a direct evaluation of its specific contribution to the observed salivary microbiome patterns. The relationship between breastfeeding practices and oral microbial diversity in infancy remains insufficiently understood and deserves more investigations.

Regarding cytokine analyses, among mothers, no differences were observed in pro- or anti-inflammatory protein levels, which may reflect the relative stability of the inflammatory profile during the evaluated periods, consistent with the overall homogeneity of the cohort. In contrast, infants showed only one statistically significant difference over time, with IL-1β levels differing between 1 and 6 months. Interleukin-1β (IL-1β) is an important inflammatory cytokine that acts through receptors expressed on multiple cell types and is therefore involved in the regulation of pro-inflammatory processes and has been studied as a relevant biomarker of immune function in infants [38]. Although salivary cytokines have been less frequently investigated in infants due to limited sample volume and lower physiological concentrations, they represent promising biomarkers of mucosal immune function [39,40].

Pro-inflammatory cytokines IFN-γ (at 1 and 3 months) and IL-12 (at 1, 3, and 6 months) showed lower levels in mothers and higher levels in infants, whereas IL-1β (at 3 and 6 months) and IL-8 (at 1, 3, and 6 months) were increased in mothers and decreased in infants. Cytokines such as IL-1β, IL-6, IL-16, IFN-γ, TNF-α, and IL-8 can be significantly influenced by oral inflammatory conditions, including dental caries and periodontitis [41,42]. This association is particularly relevant to our cohort, as a considerable proportion of the pregnant women presented with pronounced oral health issues, including 25.9% exhibiting more than two unhealthy periodontal sextants and 29.7% presenting a high caries experience (DMFT ≥ 8). In addition, higher salivary levels of TNF-α and IL-1β have been reported in pregnant women with obesity, suggesting that systemic and oral alterations may differentially modulate the inflammatory profile of mothers and infants [41].

Regarding anti-inflammatory cytokines, the levels of IL-10 (at 1 month) and IL-13 (at 1 and 3 months) were lower in mothers compared to their infants. This pattern aligns with previous studies indicating that pregnancy typically induces a shift toward a pro-inflammatory state, characterized by decreased anti-inflammatory and elevated pro-inflammatory mediators. Furthermore, consistent with our clinical observations, these immunological shifts have been shown to strongly correlate with altered periodontal parameters and specific salivary microbiota profiles in pregnant women [43].

Although this study offers unique strengths—including longitudinal monitoring from late pregnancy through six months postpartum, detailed characterization of feeding practices and predominantly middle-class socioeconomic factors, and comprehensive oral health profiling—it also has limitations. By six months, infants undergo dramatic dietary shifts and primary tooth eruption, both of which are key drivers of microbial community assembly. While we observe a tendency toward greater similarity of microbial profiles by this stage, these early-life physiological changes may actively shape the trajectory and timing of oral microbial establishment. Our cohort is relatively small and homogeneous with respect to feeding and delivery modes. The predominance of cesarean deliveries and breastfeeding limited the assessment of these factors as determinants of early oral microbiome development and may restrict the generalizability of our findings. Furthermore, the use of a dichotomized CPI did not capture differences in periodontal disease severity and may have masked clinically relevant associations. Still, considering that hypertensive disorders (which affect 8.04% of pregnancies in American region) were excluded, the predictive biomarkers or baseline profiles identified here may not represent the immunological and microbial reality of high-risk pregnancies. Future research involving larger, more diverse populations including hypertensive patients, varied feeding and delivery contexts, more detailed periodontal stratification, and extended follow-up will be critical to uncover the factors that govern early oral microbiome development and its potential as an early modifiable determinant with lifelong health implications.

In conclusion, our study is consistent with the concept of initial microbial assembly in infant influenced by maternal oral microbiome and early-life microbial imprinting, may reflect close maternal–infant interactions and shared environments. While maternal oral microbiome presents a maintenance of a consistent maternal salivary microbiome profile, infant`s salivary microbiota undergoes dynamic maturation, progressively enhancing complexity and merging toward the

maternal profile. This study advances our understanding of initial oral microbial ecosystem establishment and underscores the profound influence of caregiver oral health status on infant oral microbiome trajectory, providing key insights for future mechanistic studies on early life oral health programming.

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Apêndices: Material Suplementar.

3. CAPÍTULO 2: Artigo aceito para publicação:

"Longitudinal characterization of maternal and infant salivary proteomic profiles across pregnancy and early infancy"

A saliva contém proteínas que podem fornecer informações importantes sobre a nossa saúde. Neste estudo, acompanhamos 20 mulheres durante a gestação e após o nascimento de seus bebês para investigar as proteínas presentes na saliva das mães e das crianças com um mês de vida. Encontramos semelhanças entre os perfis salivares dos três grupos, incluindo cinco proteínas presentes tanto nas mães quanto nos bebês. Entre elas, destacaram-se proteínas da família das amilases, envolvidas principalmente na digestão dos carboidratos. Os resultados ampliam o conhecimento sobre as características da saliva na relação mãe-bebê e apontam para seu potencial como uma ferramenta simples e não invasiva para futuras pesquisas e estratégias de acompanhamento da saúde materno-infantil.

Este artigo foi aceito para publicação pela revista científica "*BMC Pregnancy and Childbirth*" - ISSN: 1471-2393. Fator de impacto (*JCR ou Scopus Citescore*): 4.8. *Highest percentile* (JCR ou Scopus): 78%. Qualis da revista de acordo com o percentil: conceito A2 na Medicina 1.

Longitudinal characterization of maternal and infant salivary proteomic profiles across pregnancy and early infancy

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Abstract

Objective: To investigate the salivary proteomic profile in the mother-baby dyad, considering the third trimester of pregnancy. **Materials and Methods**: Twenty pregnant women (PW) in the last trimester were selected and followed up. The PW were examined to assess caries experience (DMFT) and periodontal condition (CPI). Income, education, body mass index (BMI), and dietary practices were assessed through a questionnaire. Saliva samples were collected during pregnancy and postpartum (PP), as well as from the one-month infants (IF), and analyzed using mass spectrometry. Data were evaluated by descriptive statistics and bioinformatics. **Results:** PW in the study were from mid-level socioeconomic backgrounds with a high prevalence of caries (80%) and periodontal changes (60%). The average BMI was 28.37 kg/m2 in pre-pregnancy, indicating an overweight status, and the mean weight gain by the 3rd trimester was 7.23 kg. 35-40% of PW reported high consumption of carbohydrate. Proteomic analysis identified 21, 27, and 25 proteins in PW, PP, and IF, respectively, with five proteins common to all groups. Key biological interactions were observed for salivary amylase (AMY1A, AMY1B, AMY1C), involved in carbohydrate metabolism and potentially linked to overweight. **Conclusion:** The salivary proteomic profiles of PW, PP, and IF showed notable similarities, suggesting relevant biological relationships with a potential role in mother-baby dyad health monitoring.

Keywords: *pregnant women; infant; saliva; proteomics; oral health*.

1. Introduction

Pregnancy and early childhood are periods of considerable physiological and behavioral changes, critical stages for establishing healthy habits, and unique opportunities for health-promoting interventions. Increased progesterone and estrogen in maternal circulation during pregnancy can lead to immune system modifications[1]. Metabolic changes can substantially increase food intake (quantity and frequency), particularly concerning carbohydrate consumption, which may be 15% higher than during the non-gestational period, favoring overweight or obesity[1].

Daily oral hygiene becomes difficult during pregnancy due to episodes of nausea and vomiting[2]. Hormonal changes and inadequate oral hygiene during pregnancy amplify the inflammatory response to dental biofilm, leading to gingival inflammation and an increased tooth decay, especially when combined with sugar consumption[2]. Inflammation of teeth-support tissues, pre-gestational overweight, and gestational obesity have been linked to systemic disturbances, including pre-eclampsia, premature birth, and low birth weight[3].

Prenatal dental care is crucial for establishing appropriate preventive and therapeutic strategies and assuring the mother's good oral health regarding dental caries and periodontitis, for example[2]. More importantly, educational counseling during pregnancy directly impacts the prevention of early childhood caries (ECC)[4].

Saliva is a vital biological fluid and a source of biomarkers, due to its wide range of bioactive proteins and peptides [5]. Interestingly, its capacity to reflect serum characteristics related to systemic diseases has already been reported[5,6]. As for its diagnostic potential and non-invasive assessment, analyzing salivary proteins is a broad research topic of increasing interest. Over 2,000 proteins and peptides have been detected in saliva, playing diverse roles in various biological processes within the oral cavity and the human body [5]. Advanced proteomic techniques, such as mass spectrometry, enable the identification of numerous biomarkers, which are valuable tools for early diagnosis, disease monitoring, and treatment [7–9].

Differences in the human salivary proteome have been highlighted in pregnant women[9], preterm newborns [10], and infants[11]. Although limited, salivary proteomics studies in pregnancy have identified potential biomarkers, such as elevated Annexin-1 levels in women with gingivitis [12] and Metallothionein 2 (MT2A) as a possible indicator of preterm birth[10], for

example. Manconi et al. (2013)[11] used RP-HPLC–ESI–MS analysis to quantify proteins and peptides, revealing marked changes in the salivary proteome of children from birth to 48 months, and, except for statherin and histatin-3, major salivary proteins showed their lowest concentrations in the 0–6-month age group. Interestingly, recent literature has increasingly examined salivary cortisol and α-amylase as potential stress biomarkers [13]; nonetheless, findings in pregnant women did not yield significant results when compared to the postpartum period [14].

However, to our knowledge, no evidence is available to evaluate the salivary proteins of mothers and their early-life infants simultaneously, particularly using mass spectrometry. Our descriptive longitudinal study aims to understand potential changes in the proteomic profile of saliva, thereby elucidating the biological mechanisms that influence both oral and general health. Therefore, we aimed to investigate the salivary proteins in the mother-baby dyad, from the third trimester of pregnancy until the first month after the baby's birth.

2. Materials and Methods

2.1 Study design and population

Following the Declaration of Helsinki, this longitudinal observational study was conducted between November 2023 and July 2024, after obtaining ethical approval (Ethics Committee of the University São Francisco - protocol number: 65319322.2.0000.5514) and receiving written consent.

This study conveniently recruited 20 pregnant women and their infants from the largest Gynecology and Obstetrics Clinic of Extrema, Brazil. Extrema city is located in the south of Minas Gerais State, has ≅36.951 inhabitants, presents a human developmental index of 0.732, and optimally fluoridated tap water (0.69 mg F/L).

This study was part of a larger investigation involving 104 participants. Pregnant women (PW), in their third trimester (between 28 and 41 weeks of gestation) were eligible for inclusion if they completed salivary sample collection (flow rate >0.3ml/min [15]) and provided their infants' saliva samples at 1 month of age.

Exclusion criteria relied on use of antibiotics, corticosteroids, or antihypertensive medications during the study period or within the month preceding enrollment, and the presence of

neuromotor or communication impairments or psychiatric disorders. No participants presented hypertension, gestational diabetes mellitus, or orthodontic appliances at the time of enrollment.

Among the initially eligible participants, some declined participation, while others were lost to follow-up after delivery and therefore did not complete all study procedures (Figure S1). A total of 20 women and their infants fulfilled the study requirements and were included in this follow-up study, yielding 40 participants. Individuals were classified into three groups: pregnant women (PW group), mothers (M group—the same participants evaluated after delivery), and infants (IF group).

2.2 Assessment of pregnant women's oral health and dietary practices

The oral exam was conducted in the clinic's waiting room. The presence or absence of dental biofilm was assessed by visually examining the buccal surfaces of maxillary incisors. After that, PW had their teeth cleaned and dried with gauze (Medi House, São Paulo-SP, Brazil), and the diagnosis of dental caries (decayed, missing, and filled surfaces and teeth - DMFS/DMFT index) and periodontal evaluation (Community Periodontal Index - CPI) was performed according to the World Health Organization (WHO) criteria[16]. The diagnosis was conducted using artificial light (Sfl5540, Wat Nichia Philips, Brazil), a mirror, and a ball-ended dental probe (Indusbello, Londrina- PR, Brazil). Clinical examination was performed by one dentist, previously calibrated by a gold standard examiner, who received all the theoretical and practical instructions regarding the criteria to be used. About 10% of the sample was re-examined during the calibration process with a 1-week interval. A Kappa statistic was calculated to ensure homogeneous intra-examiner assessment, indicating excellent agreement, with a value of 0.86. Pregnant women who needed dental treatment were referred for clinical care.

Maternal dietary practices during pregnancy were assessed using a structured and simplified questionnaire recommended by the WHO to determine the frequency of consumption of specific food groups among adults [16]. Socioeconomic questions were added to the questionnaire ("What is your family income?" and "What is your education level?"). In addition, the initial and final weights of the pregnant women were collected from medical records, and the body mass index was calculated according to the WHO guidelines [17]. After the baby was born, information regarding the baby’s weight and height was assessed from the baby's information card. Information on the type of breastfeeding (exclusive, formula, or mixed) was obtained through direct interviews with

the mothers.

2.3 Saliva sample collection

Saliva from the PW was collected during the dental examination, in the morning, without any instructions regarding toothbrushing, as the presence/absence of dental biofilm was being analyzed. Postpartum women were asked to refrain from eating or brushing their teeth for 1 hour prior to saliva collection and from feeding their babies. Saliva collection from the postpartum women and their infants was performed in the morning period when they completed one month of life, corresponding to a range of 30–35 days.

At least 500 microliters of unstimulated mothers' saliva were collected by passive drooling (3 minutes) in plastic cups and transferred to microcentrifuge tubes using sterilized disposable syringes. The babies' saliva was collected using SalivaBio's Infant Swab (SIS) Method (Salimetrics, USA). It allows an easy, clean, and less invasive collection, carried out in two steps: 1. The roll was inserted into the baby's mouth until it was soaked with saliva; 2. The roll was removed and inserted into the plastic tube. Saliva was kept refrigerated during the entire collection period. All saliva samples were transported under refrigeration to the Laboratory of Clinical and Molecular Microbiology. The samples obtained with Salivette were centrifuged at 5,500 rpm for 10 minutes under refrigeration, and then the supernatant was transferred to 1.5 ml microtubes. All samples were frozen at -80°C.

2.4 Saliva preparation for protein mass spectrometry analysis

In the Clinical and Molecular Microbiology Laboratory, samples were thawed, and the preparation was carried out on ice when applicable. Fifty microliters of 8M urea solution was added to each 100 µl saliva sample. After vortexing, the samples were heated in a dry bath at 80°C for 15 minutes to denature proteins by disrupting hydrogen bonds, thereby enhancing trypsin activity, followed by centrifugation to remove potential cellular debris. Five microliters of 100 mM dithiothreitol (DTT) were added, the samples were vortexed again and incubated at 60°C for 30 minutes. After cooling to room temperature, the samples were centrifuged again, and 5 µl of 300 mM iodoacetamide was added. Due to the sensitivity of iodoacetamide to light, samples were kept

in a dark box at room temperature for 30 minutes. Then, 200 µl of 50 mM Tris-HCl (pH 8) and 20 µl of trypsin solution (40 ng/µL in 50 mM NH_4HCO_3) were added, to achieve 1:100 enzyme-protein ratio. Samples were vortexed and incubated overnight at 37°C for digestion. To stop the enzymatic reaction, 20 µL of 5% trifluoroacetic acid (TFA) was added. Afterward, the samples were frozen at -80°C, lyophilized for 2 hours, and stored at -20°C. Samples were diluted in ultrapure water containing 0.1% formic acid for mass spectrometry analysis, which was performed within 1 hour[18]. Sample concentrations were normalized based on total protein content determined by the Bradford assay (BioRad, Hercules, CA, USA).

2.5 Salivary proteins for mass spectrometry analysis

Mass spectrometry is an advanced analytical technique that identifies molecules based on their mass-to-charge ratio (m/z). For protein analysis, 10 µl aliquots of prepared saliva samples were applied to a C18 column (130Å, 1.7 µm, 2.1 mm x 50 mm, ACQUITY UPLC BEH, Waters Co). The eluted peptides were automatically injected into a Q-ToF Xevo G2-XS mass spectrometer (Waters Co., USA). Elution was achieved by 2 minutes of wash with 0% B to remove salt and no retained compounds (no addressed to the mass spectrometry), and then a linear gradient (1-40% B; A = 0.1% formic acid; B = 90% acetonitrile in 0.1% formic acid) at 0.2 µl/min for 90 min. Spectral data were collected in positive ionization mode (200-1800 m/z) with a resolution of 40,000 FWHM at a mass-to-charge ratio (m/z) of 500. Parameters were set at 3 kV capillary voltage, 150°C source temperature, 350°C desolvation temperature, and 600 L/h desolvation gas flow. Argon collision energy was used for MS/MS analysis, with intensity adjusted based on peptide charge. MassLinx 4.2 software was used for instrument control and data acquisition[18].

2.6 Statistical analysis and data visualization

Population characteristics, including maternal age, BMI, gestational weight gain, periodontal/dental caries index, infant weight and length at birth/1 month, and household income, were assessed using descriptive statistics. The proteome data were analyzed qualitatively using Peaks Studio V.7.0 software (Bioinformatics Solutions Inc., Waterloo, ON, Canada) alongside descriptive statistical methods. The proteins that we identified agreed with the Peaks Studio software statistical parameters. For protein identification, raw mass spectrometry files were

imported into Peaks Studio, referencing the International Protein Index (IPI) and a custom database from UniProt (www.uniprot.org) based on the terms "secreted proteins" and "human." Key parameters included MS and MS/MS tolerance set to 2 ppm, variable modifications for deamination at N and Q, N-terminal acetylation, oxidation at M, a maximum of three missed cleavages, and up to three variable post-transcriptional modifications (PTMs) per peptide. Non-specific cleavage was set as both, with a false discovery rate (FDR) of ≤0.5%. Proteins with a score ≥20 and at least one unique peptide were included in the study.

Identified proteins were examined using Venn Diagrams for comparative expression with CNB-CSIC Venny 4 (an online tool) (Fig. 1). Gene ontology was performed using the UniProt database (Fig. 3-A and Fig. 3-B), and biological pathway enrichment (Fig. 2) was analyzed via the String program (version 11.5, online tool).

3. Results

3.1 Participants' characteristics

The characteristics of pregnant women, including age, body mass index, dental caries index, income, education, and dietary practices, are presented in Table 1. Sixty percent of third-trimester pregnant women had visible dental biofilm on their maxillary upper incisors, and approximately eight teeth were affected by caries lesions per woman. The major component of the dental caries index was dental restorations (69%). Based on the CPI index, 40% of the pregnant women had all sextants in healthy conditions. Considering the worst condition identified in at least one sextant, bleeding was observed in 20% of the pregnant women, 30% had dental calculus, and 10% had periodontal pockets of 4-5 mm or sextant exclusion due to the absence of teeth (less than two).

Regarding feeding practices in the first month postpartum, 60% of the babies were exclusively breastfed, 30% combined breastfeeding with formula, and 10% were exclusively formula-fed. Although 90% of pregnant women report consuming fresh fruit at least once a week, the frequency of daily consumption was low (35%).

Table 1. Populations' characteristics.

	Mean	Standard Deviation	Median	Interquartile Range
PW Age (years)	29.63	± 8.31	29	± 8.50
Pre-pregnancy *BMI* (Kg/m^2)	28.37	± 6.29	28.26	± 5.92
Weight gain during pregnancy (Kg)	7.23	± 4.34	7.20	±5.50
PW Dental Caries index *(DMFT/DMFS)*	7.94 / 13.63	7.30 / 15.59	8 / 10	6.50 / 6.50
Infant's birth weight and length (Kg/cm)	3.02 / 48.42	0.49 / 1.39	3 / 48	0.58 /2.25
Infant's weight and length after 1 month (Kg)	4.03 / 53.70	0.85 / 2.62	4.30 / 53	1.03 / 4.25
Income (USD)	695	522	519	329
	Highest education degree			
	Number	**%**		
Middle school	2	10		
High school	13	65		
College	5	25		
	Consumption more than once a week			
	Number	**%**		
Cookies and Cakes	8	40		
Sweets and candies	4	20		
Soft drinks or lemonade	4	20		
Tea with sugar	7	35		
Coffee with sugar	8	40		

BMI: Body Mass Index; DMFT: decayed, missing, or filled teeth (permanent); DMFS: decayed, missing, or filled surface.

3.2 Proteomic profile

Proteomic analysis identified 55 distinct proteins (Table S1 - Supplementary Table 1) in the saliva samples of the studied population, and Figure 1 illustrates the overlapping proteins among the groups. Eleven, 14 and 17 proteins were detected only in groups PW, PP, and IF, respectively. Five proteins were obligatorily shared among all groups: salivary amylases (AMY1A, AMY1B, AMY1C) and pancreatic alpha-amylases (AMY2A, AMY2B). Three other proteins (Albumin, Actin cytoplasmic 1, and Lysozyme C) were commonly identified in both the PP and the IF groups. Only five proteins (IGH@ protein, Immunoglobulin heavy chain variant, Immunoglobulin heavy constant alpha 1, Immunoglobulin heavy constant alpha 2, and SNC66 protein) were shared between the PW and PP groups. No protein was concomitantly found in the PW and the IF groups.

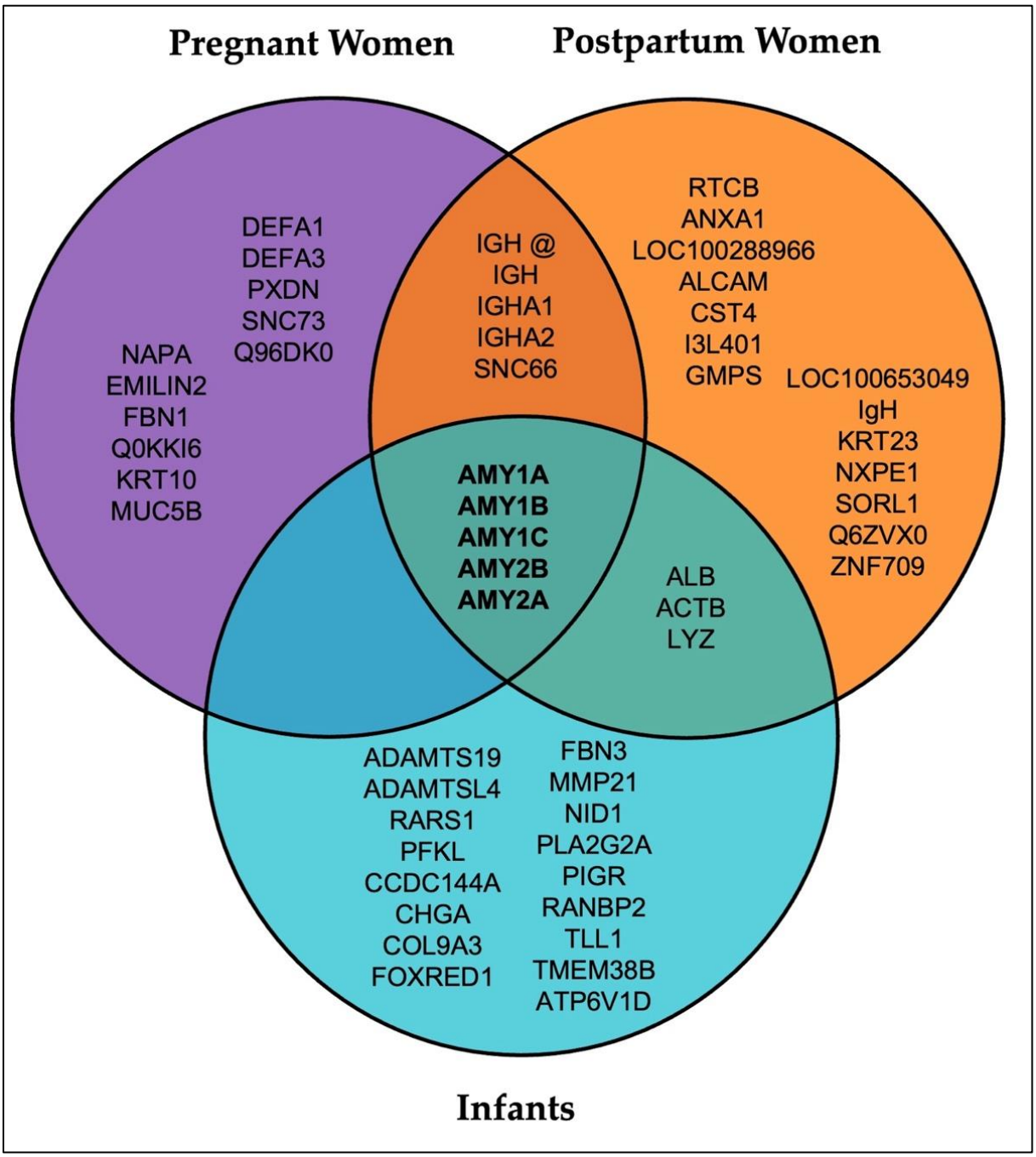


Figure 1: Distinct and shared proteomic signatures across pregnant women, postpartum women, and infants. Venn diagram illustrating the distribution of identified proteins among three groups: Pregnant Women (PW), Postpartum Women (PP), and Infants (IF). Each circle represents the proteome profile of a specific group, highlighting proteins that are exclusively detected in one group (outer regions) and those shared among two or all three groups (overlapping regions). Notably, five amylase isoforms (AMY1A, AMY1B, AMY1C, AMY2A, AMY2B) were the unique proteins shared across all groups.

Biological Pathways (Fig. 2) presents the interaction maps of proteins identified in each group, representing the molecular networks specific to pregnant women, postpartum women, and infants. In these networks, nodes represent individual proteins, while the connecting lines (edges) denote established or predicted functional associations. Generally, distinct protein compositions and interactions were found among the PW, PP, and IF groups. However, a notable pattern of solid interactions between the same protein's genes deserves to be highlighted: amylases (AMY1A, AMY1B, AMY1C, AMY2A, and AMY2B).

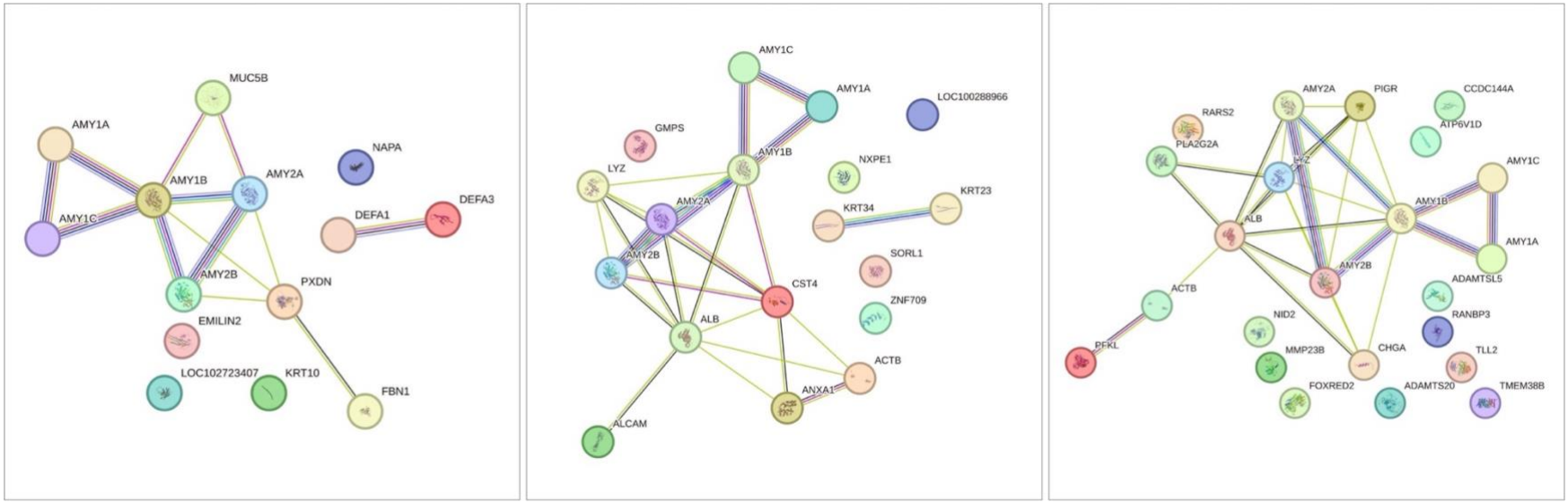


Figure 2. STRING biological pathway according to the studied groups. Protein-protein interaction networks of the identified proteins in (A) pregnant women, (B) postpartum women, and (C) infants. Each node represents a protein, and edges indicate known or predicted interactions. Network statistics: (A) number of nodes: 14; number of edges: 13; average node degree: 1.86; avg. local clustering coefficient: 0.607. (B) number of nodes: 18; number of edges: 25; average node degree: 2.78; avg. local clustering coefficient: 0.645. (C) number of nodes: 23; the number of edges: 26; average node degree: 2.26; avg. local clustering coefficient: 0.414.

Figures 3 display the Gene Ontology (obtained from the UniProt database) according to molecular function (A) and biological process (B). Gene ontology (GO) is a taxonomy that statistically determines the sets of genes involved in the same biological processes, functions, or localizations.

Regarding molecular function (GOMF), no one was common to all three groups. Still, several were shared between the pregnant women (PW) and postpartum (PP) groups with the same frequency (alpha-amylase activity, calcium ion binding, chloride ion binding, antigen binding) as shown by the horizontal bars in the gene ontology (Fig. 3). In contrast, the most prevalent molecular functions in the infant (IF) group were carbohydrate catabolic process. Oligosaccharide metabolic process, another function related to carbohydrate digestion, was also identified in this group. Interestingly, we also observed functions associated with innate immunity, including extracellular matrix organization, defense response to Gram-positive bacteria, inflammatory response, and killing of cells from other organisms (Fig. 3-A).

Analysis of the biological process (GOBP) patterns revealed differences among all studied groups, with only one process, the oligosaccharide metabolic process, shared by all three groups, as indicated by the horizontal bars in the Gene Ontology (Fig. 3-B). The carbohydrate catabolic process was the most prevalent biological process. It appeared only for PP and IF, while the most prevalent biological process for pregnancy was the carbohydrate metabolic process. The inflammatory response was shared between the PW and IF groups. Furthermore, in the IF group, processes related to immune response (defense response to Gram-negative bacteria, killing of cells of another organism) deserve highlighting.

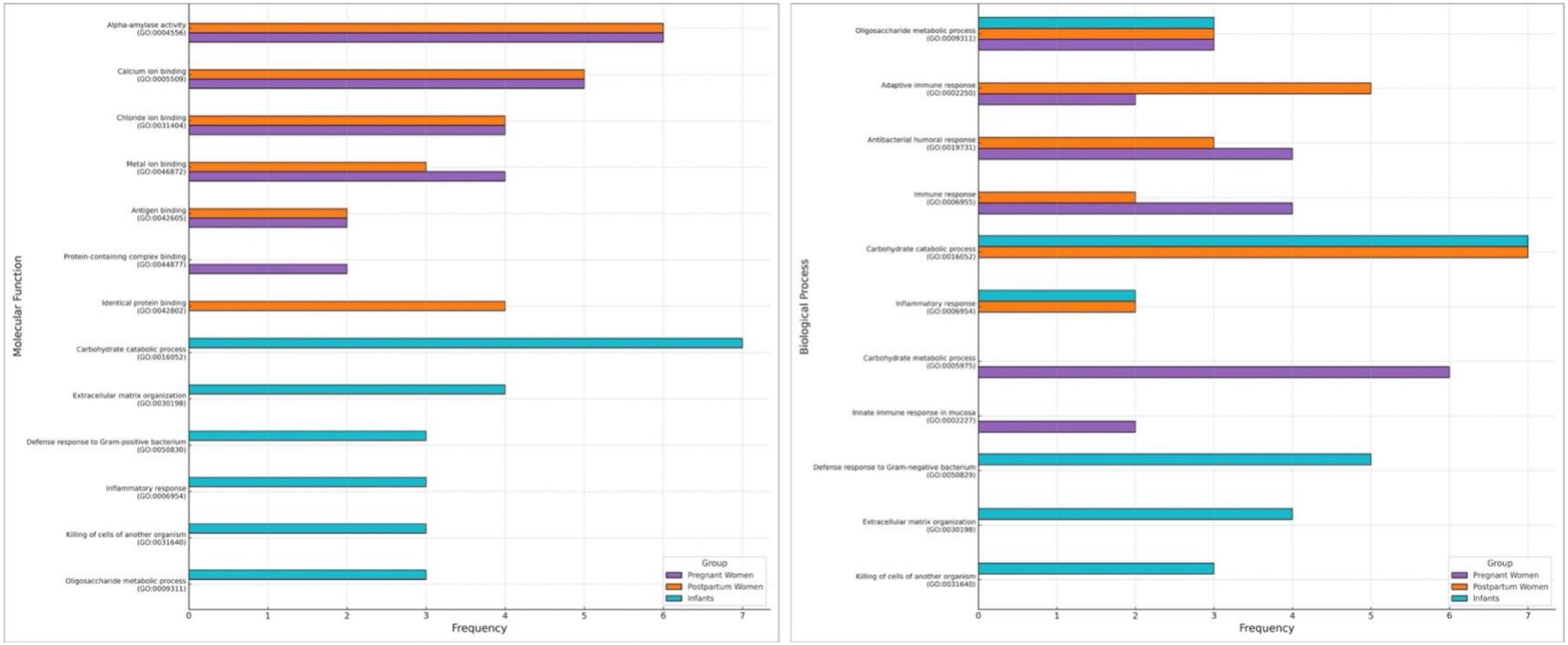


Figure 3: A) Gene-Ontology (GO) considering the identified proteins' molecular function. Gene Ontology (GO) analysis reveals the functional categorization of proteins found in pregnant women (purple), postpartum women (orange), and infants (blue). Bars represent the frequency of proteins associated with each molecular function, highlighting both shared and group-specific roles. **B) Gene-Ontology (GO) considering the identified proteins' biological process.** Gene Ontology (GO) analysis reveals the process categorization of proteins found in pregnant women (purple), postpartum women (orange), and infants (blue). Bars represent the frequency of proteins associated with each biological process, highlighting both shared and group-specific roles.

4. Discussion

It is well known that saliva is a rich biological fluid and a potential source of biomarkers. The results of the present study highlight remarkable interrelationships in the proteomic profile of pregnant women (PW), postpartum women (PP), and their infants (IF). Unstimulated salivary samples seemed to be a good choice for proteomic analysis[11,19]. In the PW, we identified 21 different proteins, similar to the findings of Balan et al., 2021[20] (28 proteins) and comparable to pregnant women in other pregnancy periods (47 and 18 proteins for early and mid-gestational stages, respectively) reported by Dey et al., 2020[21]. Interestingly, these authors[21] pointed to an inverse relationship between the number of proteins and gestational age, i.e, the higher the gestational period, the lower the number of proteins found. Concerning the PP and IF groups, age-related variations in the salivary proteome have been reported in preterm newborns and infants [11,22], underscoring the influence of developmental stage on protein expression; however, these findings were obtained using different techniques.

Interestingly, in all groups of the present investigation, amylase 1B (AMY1B), a salivary isoenzyme synthesized by all three major glands, with the parotid gland being the predominant source, was identified. Previous research identified five alpha-amylase isoforms in obese women during late pregnancy, noting a ten-fold upregulation in those with concurrent periodontitis [23]. Furthermore, within a similar cohort, the specific presence of AMY1B was associated with severe gingival bleeding on probing (>50%) [24]. AMY1B is involved in carbohydrate metabolism and influences appetite and weight regulation[25]. In general, amylases are secreted proteins capable of hydrolyzing 1,4-alpha-glucosidic bonds in oligosaccharides and polysaccharides, thereby catalyzing the initial step in the digestion of starch and glycogen. Additionally, our study identified pancreatic amylase, represented by the gene codes AMY2B and AMY2A, which acts in the small intestine[25]. In line with the above findings, the women's initial BMI before pregnancy, indicated that they were overweight. In the third trimester, the mean weight gain indicated that the pregnant woman persisted in the overweight category. Pregnancy weight gain is linked to eating habits [26]. It was already reported that gestational weight gain increased by 1.33 kg for each 1-percentage-point rise in the proportion of dietary energy derived from ultra-processed foods [26]. Higher consumption of saturated fats and ultra-processed foods contributes also to greater postpartum weight retention [27]. Of interest, 35-40% of the pregnant women in our study reported consuming large quantities of sweet cookies and cakes, as well as sugar-sweetened tea and coffee. These foods

are rich in sucrose and contain empty calories, contributing to increased weight gain during pregnancy and promoting unhealthy conditions in the oral cavity, such as caries lesions and gingivitis (precursor to periodontitis), due to biofilm accumulation [28]. High gestational weight gain has been linked to an elevated risk of dental caries even in their offspring [29]. Additionally, diet has a direct impact on the salivary protein composition [30].

Salivary amylases (AMY1A, AMY1B, AMY1C) and pancreatic alpha-amylases (AMY2A, AMY2B) form an interesting relationship that can be observed in the PW, PP, and IF groups, as shown in the STRING biological pathway, likely due to the carbohydrate-rich dietary profile, including lactose. The interplay between dietary carbohydrate intake and AMY1 polymorphisms affected the incidence of type 2 diabetes in Korean women [31]. This dietary link is further supported by a fish model, where increased carbohydrate consumption triggered a significant rise in amylase levels [32]. Perhaps our study provides a first insight into how protein profiles associated with overweight/obesity appear in pregnant women, mothers, and their babies. Although the infant's birth weights were average (higher than 2.5), and most of them were breastfed, their nutritional status warrants a follow-up investigation.

A broad view of the proteomic profile was evident in the Gene Ontology of salivary proteins. Gene Ontology is represented by the letters "GO" and is followed by a number, in a computer-readable format. It consists of sets of genes connected to functional molecules, such as proteins, which are statistically classified basically according to biological processes (GOBP) and molecular function (GOMF), which were included in the present study. GOMF analysis reveals similarities in terms of composition between the pregnant women (PW) and postpartum (PP) groups. Alpha-amylase activity (GOMF) was the most prominent in both groups, highlighting the role of amylases in these biological profiles. Calcium, chloride, and metal ion binding were the most prevalent molecular functions observed among these groups.

Calcium ion binding was the second most prevalent molecular function in PW and PP groups (GOMF). It is connected to calcium ion storage activity. Calcium ion plays a crucial role in salivary electrolyte dynamics and are essential for maintaining the integrity of dental tissues. It actively remineralizes carious lesions and promotes the supersaturation of minerals in saliva, thereby strategically minimizing the damage caused by saccharolytic bacteria[33]. Considering that the PW and PP groups involved the same individuals at distinct stages and that these women exhibited a

high prevalence of caries lesions and a significant presence of visible biofilm, it can be inferred that essential changes in calcium ion bioavailability are occurring in the oral environment, favoring the expression of calcium-binding proteins. Interestingly, it has already been demonstrated that a more disorganized structure of the biofilm (immature) provides a significant absorption of calcium from saliva to the tooth-enamel interface, reducing its bioavailability in saliva, while a more organized biofilm (mature) does not, allowing more calcium availability in saliva[34]. Interestingly, in a study involving Brazilian pregnant women with obesity and periodontitis, a calcium binding protein (protein S100-A8) was found to be up-regulated in the saliva, with functions involved in inflammatory mechanisms [23].

Chloride ion binding (GOMF) is the third most prevalent molecular function shared by the PP and PW groups, referring to the ability of a protein to interact selectively with chloride ions (Cl). This interaction is crucial to many biological processes, including osmotic balance and the transport of ions. Most chloride anions are free, but some bind to proteins, influencing their spatial structure and transformations. Its functions include the regulation of enzyme activities and the prevention of infection [35]. The chloride ion binding (GOMF) connects one of the most essential transport and storage proteins in human blood plasma, albumin, to the oxygen-carrying protein in erythrocytes, hemoglobin[35]. Processes linked with infection prevention and good oxygen carriage are vital to human health.

Interestingly, metal ion and antigen binding (GOMF) are the last molecular functions common to PW and PP groups. While antigen binding is equivalent in the two groups, metal ion binding is more intense in the PW group. Metal ion binding is the ability of proteins to bind to metal ions in general (calcium, zinc, iron, copper), and antigen-binding molecules can bind to foreign antigens. This makes sense since binding to metal ions helps regulate enzymatic activity/defense against microorganisms[36], and binding to antigens induces or regulates the immune response. Bacteria can adhere to salivary proteins adsorbed on the tooth's surface or to free salivary components, becoming agglutinated and removed from the oral cavity[36] by swallowing or coughing, thereby preventing oral colonization. This mechanism is crucial in protecting against systemic infection[37,38].

Concerning the infant (IF) group molecular functions and biological processes, carbohydrate catabolic processes (GOMF, GOBP) predominated, and oligosaccharide metabolic processes were

also notable. In the early life, digestible carbohydrates are primarily consumed as lactose, which brush-border lactase in the small intestine hydrolyzes into glucose and galactose for absorption via sodium-glucose cotransporters [39,40]. In contrast, breast milk oligosaccharides are non-digestible and pass intact into the large intestine. There, they act as prebiotics to selectively stimulate beneficial microbiota, such as Bifidobacterium, ultimately promoting colonic health, modulating immune function, and lowering susceptibility to infection [40]. Thus, the fact that carbohydrate catabolic processes and oligosaccharide metabolic processes were expressive in the present study may indicate a salivary profile adapted to sugar metabolism; following a milk-based diet, particularly breastmilk, consumed by 60% of the infants. These metabolic pathways are crucial during the neonatal period, as they support energy production and influence immune development and early microbiota composition [41].

Other molecular functions and biological processes, such as extracellular matrix organization, killing of cells from another organism, and defense responses to bacteria, deserve highlighting in the infant group, all of which are associated with innate immunity. This aligns with the early postnatal period, characterized by immune immaturity and a heavy reliance on maternal support, particularly through breast milk, which is rich in antibodies, cytokines, and oligosaccharides (HMOs) [41]. According to Hibel et al.[42], salivary secretory IgA levels in one-month-old infants reflect maternal levels but decline sharply (by 70%) at 3 and 6 months before stabilizing. Enriching the immune-related pathways in infant saliva might reflect the transition from maternal to self-derived immunity, corroborating a gradual maturation of the infant's mucosal immune system.

Still, the carbohydrate catabolic process was the most prevalent biological process found in the IF and PP groups, followed by the carbohydrate metabolic process, observed in the PW group (GOBP). These findings reinforce the high carbohydrate intake reported by the pregnant women in our study. Such dietary habits during pregnancy may influence maternal metabolism and shape the biochemical and microbial environment to which the infant is exposed. The molecular echoes of mothers' feeding practices, reflected in the infant salivary proteome, suggest a potential vertical correlation, where maternal nutritional behavior may modulate early-life metabolic signatures and impact early metabolic programming [43]. This observation supports that maternal-infant biological relationships are beyond genetics.

Among the biological processes (GOBP) identified, the oligosaccharide was the only one present in all three groups (PW, PP, and IF), supporting a critical link between maternal and infant physiology. Notably, variations in the concentration of human milk oligosaccharides (HMOs)[43] have been associated with maternal obesity. In a previous study, maternal BMI (one month postpartum) also correlated with the abundance of specific HMOs [44], which are known as key prebiotic agents, as already mentioned above. Therefore, the shared oligosaccharide metabolic process in the maternal-infant dyad suggests that maternal metabolic status may influence the proteomic profile, an insight that warrants further investigation.

The present longitudinal study, involving mothers and very young babies, has several strengths, including its ability to track multiple variables, which provides a comprehensive view of early development. On the other hand, the investigation has limitations. The pregnant women were from mid-level socioeconomic backgrounds, as indicated by their income and education levels. A minority were free of caries or had all the sextants of teeth in healthy conditions. This way, a sample comprising participants with different socioeconomic backgrounds, with distinct feeding practices, and more detailed methods for the oral examinations, could bring additional information. Measuring individual protein levels would strengthen the clinical relevance of these findings; however, this was limited by the equipment's analytical capacity, which can only detect the most abundant proteins. As this is a preliminary study, further research with larger cohorts and mechanistic approaches is required to validate the proposed associations and clarify the functional role of salivary protein dynamics in the metabolic health of the mother–infant dyad.

In conclusion, this longitudinal salivary proteomics study reveals similarities in the proteomic profiles among pregnant women, mothers, and their infants, highlighting relevant biological relationships. The primary associations are marked by salivary alpha-amylase protein, which is consistently linked to greater dietary carbohydrate intake.

Declarations

Ethics approval and consent to participate: The study was conducted in accordance with the Declaration of Helsinki and approved by the Ethics Committee of the University São Francisco - protocol number: 65319322.2.0000.5514. All participants (or their legal guardians) provided written informed consent prior to participation in the study.

Consent for publication: Written informed consent for publication was obtained from the parents or legal guardians of all participants.

Availability of data and materials: All data are available in the Repositories ProteomeX-change_PXD063678 and jPOST_JPST003789, as well as in Supplementary Table 1.

Competing interests: The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Author`s Contributions: Conceptualization: TP and CC; Data curation: CC and EL; Methodology and investigation: CC and EL and JS; Formal Analysis: CC, JS, RM, and TP; Project administration: TP; Supervision: TP; Writing—original draft and review & editing: CC, CD, RM, VP, and TP. All authors read and approved the final manuscript.

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Apêndices: Material Suplementar.

4. CONCLUSÃO

Os estudos desenvolvidos nesta tese revelam que as gestantes incluídas apresentaram perfil socioeconômico médio, elevada prevalência de cárie dentária e alterações periodontais, além de consumo frequente de bebidas adoçadas com açúcar, como chá e café. Além disso, demonstrou-se pela primeira vez na literatura similaridades qualitativas entre os perfis proteômicos salivares de gestantes, mães e seus respectivos bebês, com destaque para proteínas da família das amilases, ampliando o conhecimento disponível sobre biomarcadores salivares na díade mãe–bebê. Estas semelhanças podem ser promissoras para estratégias de controle/modulação de doenças e promoção de saúde materno-infantil, especialmente quanto a sua possível associação com práticas alimentares e excesso de peso, embora estudos quantitativos e mecanísticos sejam necessários para esclarecer sua relevância biológica e clínica.

Além disso, a presente tese apresenta resultados consistentes com o conceito de compartilhamento microbiano materno-infantil nos primeiros meses de vida e descreve diferenças marcantes nos perfis de citocinas salivares entre mães e lactentes ao longo do acompanhamento. Enquanto o microbioma oral materno permanece amplamente estável, a microbiota salivar infantil passa por uma maturação dinâmica, apresentando sucessão ecológica dinâmica, com aumento progressivo da complexidade e maior similaridade em relação ao perfil materno aos seis meses de vida.

Em conjunto, estes resultados ampliam a compreensão sobre o estabelecimento inicial do ecossistema oral na díade mãe–bebê e reforçam a importância da saúde bucal materna como componente relevante do contexto biológico compartilhado durante a primeira infância. Tais achados fornecem subsídios para futuras investigações longitudinais, quantitativas e dos mecanismos biológicos subjacentes sobre microbioma, biomarcadores salivares e desenvolvimento da saúde bucal infantil.

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ANEXOS

Anexo 1: Aprovação do Comitê de Ética em Pesquisa da Universidade São Francisco (CEP/USF).

UNIVERSIDADE SÃO FRANCISCO-SP

PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: Saúde bucal, microbiota salivar e dieta de gestantes e sua influência sobre o estabelecimento de microrganismos cariogênicos em seus bebês: estudo longitudinal

Pesquisador: Camila Lopes Crescente
Área Temática:
Versão: 1
CAAE: 65319322.2.0000.5514
Instituição Proponente: Universidade São Francisco-SP
Patrocinador Principal: Financiamento Próprio

DADOS DO PARECER

Número do Parecer: 5.796.244

Apresentação do Projeto:
Projeto de grande relevância clínica, adequadamente elaborado e esclarecedor envolvendo 140 participantes, sendo 70 gestantes no período gestacional de terceiro trimestre e posteriormente ao parto, 70 bebes.

Objetivo da Pesquisa:
Tem por objetivo investigar a saúde bucal, a composição microbiológica salivar e a dieta de gestantes a fim de verificar sua influência sobre o estabelecimento da microbiota cariogênica em seus bebês .

Avaliação dos Riscos e Benefícios:
Mediante análise da metodologia, a pesquisa pressupõe risco baixo as gestantes e aos bebes. Será ofertado tratamento odontológico as gestantes que apresentarem necessidade, bem como aos bebês, quando da erupção da dentição decídua.

Comentários e Considerações sobre a Pesquisa:
Pesquisa de relevância que permitira identificar a influência da condição bucal e dieta de gestantes no período gestacional de terceiro trimestre sobre seus bebes. As gestantes serão submetidas a exame bucal para avaliação da presença de cárie, periodontite, condições inflamatórias,

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reabilitação protética e/ou com implantes. Serão coletados dados sócio demográficos, dietéticos e inerentes as condições sistêmicas gestacionais (quadros de infecções recorrentes, diabetes gestacional, peso e altura) através de questionário; assim como saliva. Assim que os bebês nascerem, o tipo de parto, se a termo ou pré-termo, o peso e altura serão registrados. Então, saliva será coletada dos bebês e mais uma vez de suas mães. Após 3, 6 e 12 meses do nascimento novas amostras de saliva serão coletadas tanto dos bebês, como de suas respectivas mães. As amostras salivares coletadas serão utilizadas para determinar a composição microbiológica através de extração de DNA e PCR em tempo real, análise do microbioma através de sequenciamento (microbial DNA qPCR array), avaliação de polimorfismos em genes associados ao sabor e para análise da composição através das concentrações de fluoreto (F-), cálcio (Ca2+), fosfato (P) e proteômica. Os dados serão avaliados por estatística descritiva e regressão de Poisson, de modo a permitir conclusão.

Considerações sobre os Termos de apresentação obrigatória:

Adequados e esclarecedores.

Conclusões ou Pendências e Lista de Inadequações:

Aprovado livre de recomendações.

Considerações Finais a critério do CEP:

APÓS DISCUSSÃO EM REUNIÃO DO DIA 01/12/2022, O COLEGIADO DELIBEROU PELA APROVAÇÃO DO PROJETO DE PESQUISAS. SENDO OBRIGATÓRIO O ENVIO DE RELATÓRIOS PARCIAIS DE SEIS EM SEIS MESES E APÓS A CONCLUSÃO DO PROJETO É OBRIGATÓRIO O ENVIO DO RELATÓRIO FINAL PARA ENCERRAMENTO DO PROJETO.

Este parecer foi elaborado baseado nos documentos abaixo relacionados:

Tipo Documento	Arquivo	Postagem	Autor	Situação
Informações Básicas do Projeto	PB_INFORMAÇÕES_BÁSICAS_DO_PROJETO_2049992.pdf	17/11/2022 18:18:56		Aceito
Outros	7LattesThais.pdf	17/11/2022 18:17:04	Camila Lopes Crescente	Aceito
Outros	6LattesCamila.pdf	17/11/2022 18:16:44	Camila Lopes Crescente	Aceito
Outros	5AutorizSecDeSaude.pdf	17/11/2022 18:16:08	Camila Lopes Crescente	Aceito
Projeto Detalhado	8Projeto.pdf	17/11/2022	Camila Lopes	Aceito

UNIVERSIDADE SÃO FRANCISCO-SP

Continuação do Parecer: 5.796.244

/ Brochura Investigador	8Projeto.pdf	18:14:33	Crescente	Aceito
Outros	4TCUD.pdf	17/11/2022 16:05:19	Camila Lopes Crescente	Aceito
Outros	3TERMODECONFIDENCIALIDADE.pdf	17/11/2022 16:05:04	Camila Lopes Crescente	Aceito
TCLE / Termos de Assentimento / Justificativa de Ausência	2TCLE.pdf	17/11/2022 16:04:26	Camila Lopes Crescente	Aceito
Folha de Rosto	1FolhaDeRosto.pdf	17/11/2022 16:03:50	Camila Lopes Crescente	Aceito

Situação do Parecer:
Aprovado

Necessita Apreciação da CONEP:
Não

BRAGANCA PAULISTA, 06 de Dezembro de 2022

Assinado por:
CARLOS EDUARDO PULZ ARAUJO
(Coordenador(a))

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Anexo 2: Questionário utilizado para coleta de informações.

Nome da gestante/mãe:_____

Nome do bebê:_____

Endereço: _____

Telefone para contato:_____

Previsão de data para o parto:_____

Local que pretende fazer a consultas com o pediatra após o bebê nascer: _____

Dados relacionados à gestante:

Peso inicial _____ Peso atual:_____ Altura:_____

Peso que ganhou na gestação_____

Escolaridade materna:

Renda familiar aproximada:

Dados relacionados ao parto e ao bebê:

- Via de parto: ( )natural ( )normal ( )cesárea - ( ) Termo ( ) Pré-termo - Semanas: _____

- Peso do bebê ao nascer: _____ 1 mês_____ 3m_____6m_____1a_____

- Comprimento do bebê ao nascer: _____ 1 mês_____ 3m_____6m_____1a_____

- Qual o tipo de amamentação?

( ) Peito ( ) Fórmula ( ) Ambos, proporção de leite materno/leite artificial:_____

Anexo 3: Questionário utilizado para avaliação de marcadores de consumo alimentar.

Você tem costume de realizar as refeições assistindo TV, mexendo no computador e/ou celular?	SIM	NÃO	NÃO SEI
Quais refeições você faz ao longo do dia? ( ) Café da manhã ( ) Lanche da manhã ( ) Almoço ( ) Lanche da tarde ( ) Jantar ( ) Ceia			
Ontem você consumiu:			
Feijão	SIM	NÃO	NÃO SEI
Frutas Frescas (não considerar suco de frutas)	SIM	NÃO	NÃO SEI
Verduras e/ou legumes (não considerar batata, mandioca, aipim, macaxeira, cará e inhame)	SIM	NÃO	NÃO SEI
Hambúrguer e/ou embutidos (presunto, mortadela, salame, linguiça, salsicha)	SIM	NÃO	NÃO SEI
Bebidas adoçadas (refrigerante, suco de caixinha, suco em pó, água de coco de caixinha, xaropes de guaraná/groselha, suco de fruta com adição de açúcar)	SIM	NÃO	NÃO SEI
Macarrão instantâneo, salgadinhos de pacote ou biscoitos salgados	SIM	NÃO	NÃO SEI
Biscoito recheado, doces ou guloseimas (balas, pirulitos, chiclete, caramelo, gelatina)	SIM	NÃO	NÃO SEI

13. Com que frequência você come ou bebe algum dos seguintes alimentos, mesmo em pequenas quantidades? (Leia cada item)

	Várias vezes ao dia	Todos os dias	Algumas vezes na semana	Uma vez por semana	Algumas vezes por mês	Raro/ nunca
	6	5	4	3	2	1
Frutas frescas	☐	☐	☐	☐	☐	☐
Bolachas, bolos, bolos recheados	☐	☐	☐	☐	☐	☐
Tortas doces, rosquinhas doces	☐	☐	☐	☐	☐	☐
Geleia, mel ou doce de leite	☐	☐	☐	☐	☐	☐
Chiclete contendo açúcar	☐	☐	☐	☐	☐	☐
Doces/balas	☐	☐	☐	☐	☐	☐
Doces caseiros (doce de abóbora, marmelada etc.)	☐	☐	☐	☐	☐	☐
Frutas em calda	☐	☐	☐	☐	☐	☐
Brigadeiro, beijinho, doces de festa	☐	☐	☐	☐	☐	☐
Limonada, coca-cola ou outros refrigerantes	☐	☐	☐	☐	☐	☐
Chá com açúcar	☐	☐	☐	☐	☐	☐
Café com açúcar	☐	☐	☐	☐	☐	☐

APÊNDICES

Apêndice I: Ficha clínica utilizada na avaliação do índice de cárie e presença de biofilme.

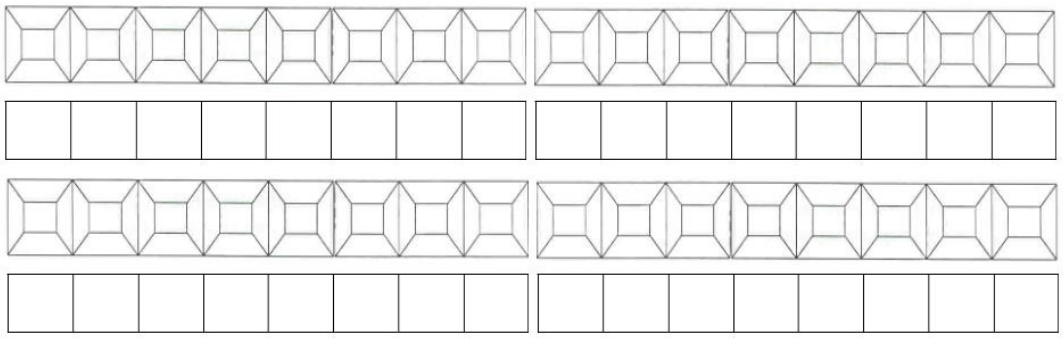
FICHA CLÍNICA – Índice de Cárie

Nome:_____

Presença de biofilme visível: ( ) sim ( ) não

Índice de Dentes Cariados, Perdidos e Obturados (CPOD)

Código		Condição
Dentes permanentes		
Coroa	Raiz	
0	0	Hígido
1	1	Cariado
2	2	Restaurado, com cárie
3	3	Restaurado, sem cárie
4	–	Perdido devido à cárie
5	–	Perdido por outra razão
6	–	Selante de fissuras
7	7	Apoio de ponte ou coroa ou *veneer* / implante
8	8	Dente não erupcionado (coroa) / raiz não exposta
9	9	Não registrado

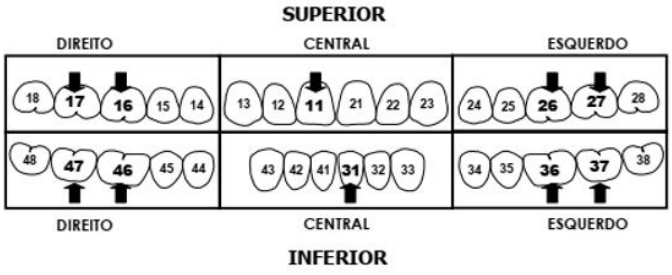


Apêndice II: Ficha clínica utilizada na avaliação da condição periodontal.

FICHA CLÍNICA – Condição Periodontal

Nome: _____

Índice Periodontal Comunitário – CPI



Códigos - São os seguintes os códigos utilizados no CPI (Figura 11):

0 - sextante **hígido**;

1 - sextante com **sangramento** (observado diretamente ou com espelho, após sondagem);

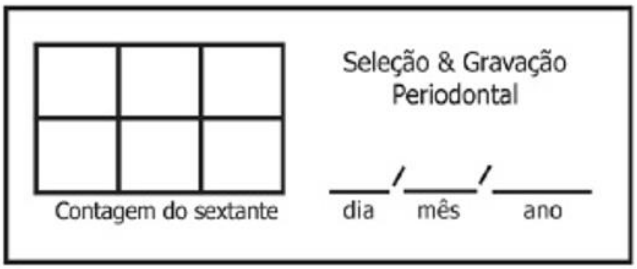
2 - cálculo (qualquer quantidade, mas com toda a área preta da sonda visível);

3 - bolsa de 4 mm a 5 mm (margem gengival na área preta da sonda);

4 - bolsa de 6 mm ou mais (área preta da sonda não está visível);

X - sextante **excluído** (menos de 2 dentes presentes);

9 - sextante **não examinado**.



Apêndice III: Material Suplementar Capítulo 1.

Supplementary Materials

DNA Extraction, Library Preparation and 16S rRNA Sequencing

Barcoding and library preparation were carried out as described [1–4]. A total of 10 ng of DNA was amplified using universal primers targeting the V3 and V4 region of the bacterial 16S RNA gene [3,4] with an average amplicon size of ~460 bp. Primer sequences contained overhang adapters appended to the 5' end of each primer for compatibility with the Illumina sequencing platform [5].

The complete sequences of the primers were: 16S Amplicon PCR Forward Primer = 5'TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG and 16S Amplicon PCR Reverse Primer = 5'GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC [6].

Master mixes contained 10 ng of total DNA, 5 μM of each primer, and 7,5 ul 2x KAPA HiFi PCR Kit (Homemade NPM). The thermal profile for the amplification of each sample had an initial denaturing step at 95°C for 3 minutes, followed by the cycling of denaturing at 95°C for 30 seconds, annealing at 55°C for 30 seconds, and a 30-second extension at 72°C (25 cycles), a 5-minute extension at 72°C and a final hold at 4°C.

Each 16S amplicon was purified using the AMPure XP reagent (Beckman Coulter, Indianapolis, IN, USA). Then each sample was amplified using a limited-cycle PCR program, adding Illumina sequencing adapters and dual-index barcodes (index i7 and index i5) (Ilumina®, San Diego, CA) to the amplicon target. The thermal profile for the amplification of each sample had an initial denaturing step at 95°C for 3 minutes, followed by a denaturing cycle of 95°C for 30 seconds, annealing at 55°C for 30 seconds and a 30-second extension at 72°C (8 cycles), a 5 minute extension at 72°C and a final hold at 4°C. The final libraries were again purified using the AMPure XP reagent (Beckman Coulter), checked on the Bioanalyzer and quantified on the SpectraMax iD3-Mode Microplate Reader [7]. Finally, libraries had concentration adjusted to 4 nM before being

pooled.

The DNA library pool was then denatured with NaOH, diluted with hybridization buffer, and heat-denatured before loading on the sequenced in Illumina NextSeq 2000 System (paired-end, 2x300). Automated cluster generation and paired-end sequencing with dual reads were performed according to the manufacturer's instructions.

References

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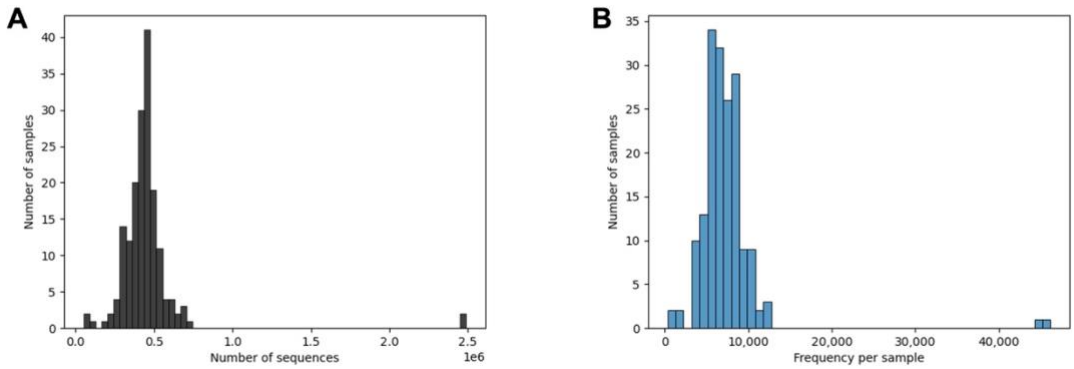
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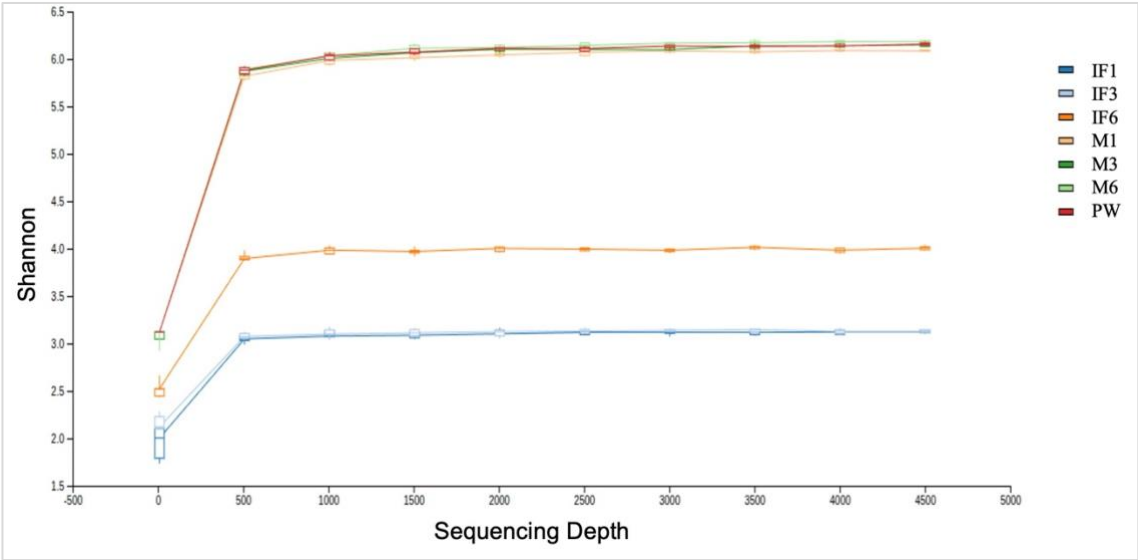
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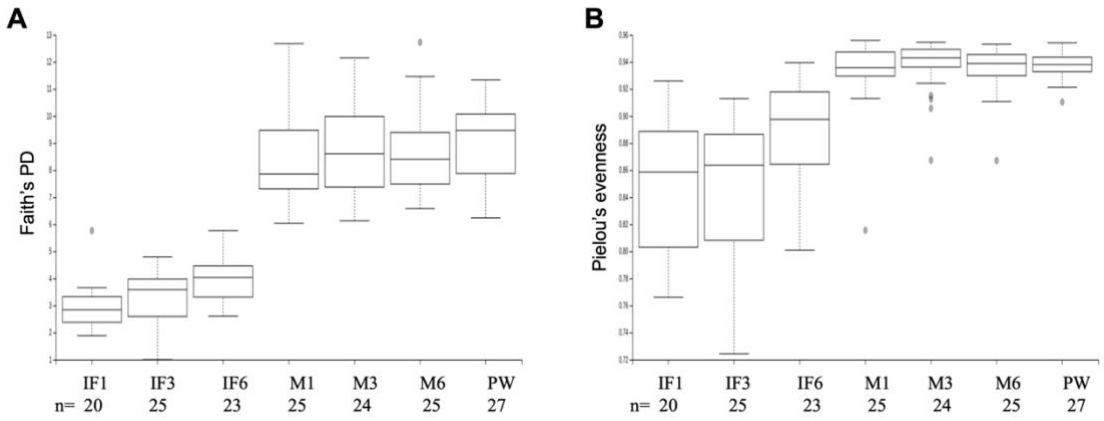
Supplementary Materials – Figures



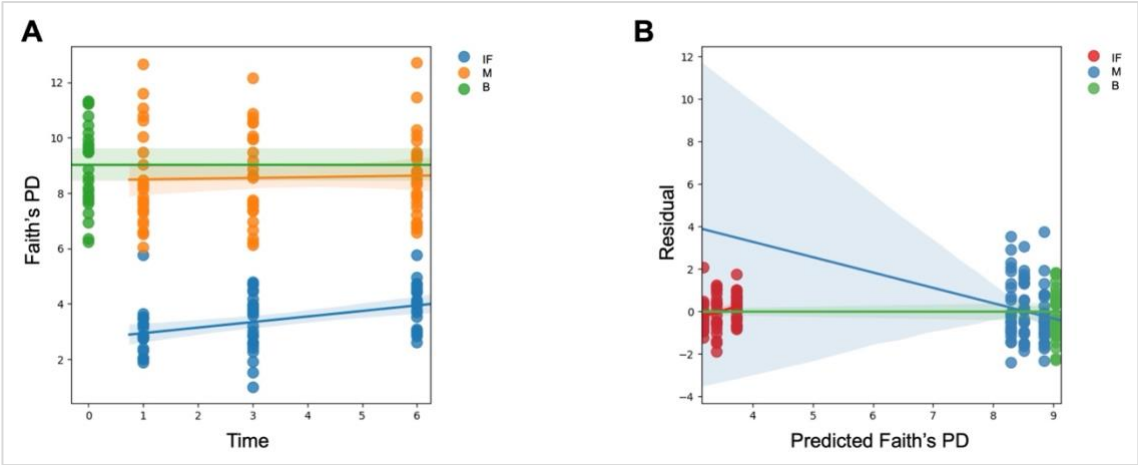
SF1. Histograms of sequencing reads and the subsequent denoised and filtered feature frequencies. A) Reads frequency histogram. B) Feature frequency histogram.



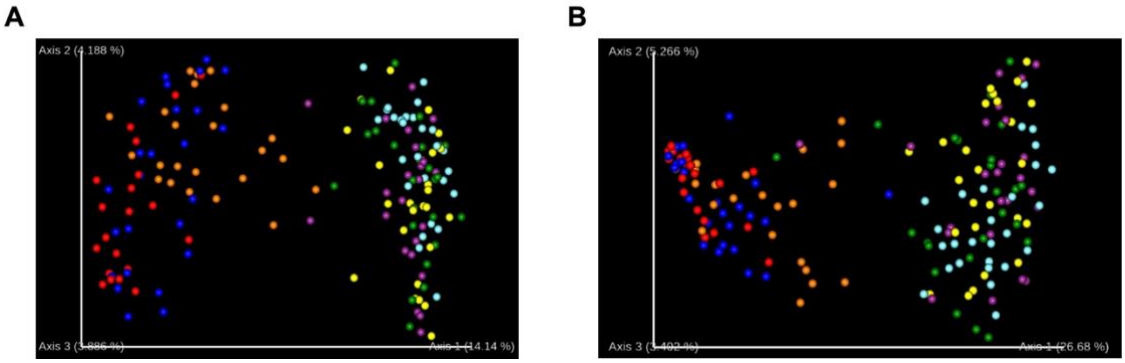
SF2. Alpha rarefaction curve, demonstrating Shannon's diversity – community richness.



SF3. Alpha diversity boxplots to demonstrate community richness and evenness. A) Faith's phylogenetic diversity (PD). B) Pielou's evenness.



SF4. Faith's phylogenetic diversity (PD) within a Linear Mixed Effects (LME) model.
A) Regression scatterplot. B) Projected residuals.



SF5. Beta diversity. A) Jaccard distance. B) Bray-Curtis distance

Supplementary Materials - Tables

ST1. Metrics of sequencing and data preprocessing.

Sample-id	Input	Filtered	Denoised	Merged	Non-chimeric	Filter2
IF1-1M	540647	33610	33242	32441	10353	10044
IF1-3M	414899	25236	24760	23635	6540	6434
IF1-6M	479078	29109	28536	26566	6721	5924
IF12-1M	280680	17041	16703	16364	1472	1265
IF12-3M	459538	28091	27724	27425	3295	2892
IF12-6M	428812	26277	25825	24958	4013	3692
IF13-1M	269653	16853	16636	15365	4014	3544
IF13-3M	101868	6268	5922	5015	2025	1372
IF14-1M	51223	3146	2420	801	454	289
IF14-3M	448807	27379	26811	25164	5738	5673
IF14-6M	403405	24609	24305	23371	6099	5811
IF16-1M	274850	16543	16276	15488	5468	4715
IF16-3M	2482963	154613	153880	150420	46169	44199
IF16-6M	390671	24125	23583	22276	3727	3663
IF19-1M	339217	21067	20819	20131	5969	5872
IF19-3M	461420	29133	28799	27949	6138	5953
IF19-6M	490880	30035	29712	28779	5859	5731
B2-3M	354623	21735	21425	20602	5096	4473
B20-3M	469567	29861	29595	28720	8876	8753
B20-6M	319750	19816	19236	18007	6284	4765
B28-1M	552306	34257	34080	33564	10694	10003
B28-3M	363994	22760	22339	21284	8111	7738
B28-6M	504690	31544	31159	29970	8825	7970
B29-1M	235160	14596	14343	13696	5203	4496
B29-3M	441922	27839	27456	26463	5276	5204
B29-6M	361478	22844	22546	21633	4109	3993
IF3-1M	49861	2986	2719	703	326	192
IF3-3M	313820	19273	18978	18201	6553	5923
IF3-6M	346547	21124	20810	20336	6471	6224
IF30-1M	402171	24738	24441	23315	4959	4797
IF30-3M	332622	21460	21144	20398	4266	4222
IF30-6M	387952	24432	23992	22950	4762	4609
IF33-3M	229375	14296	14149	13296	5206	4839
IF33-6M	390095	24681	24365	23211	5247	5052
IF34-1M	504376	30995	30570	30110	9466	9179
IF35-1M	313559	19322	19133	18930	5443	5093
IF35-3M	384550	23868	23544	22719	7309	7125
IF35-6M	359855	22760	22365	21216	3924	3658
IF37-1M	494823	30431	30044	28988	9041	8974

IF37-3M	313032	19954	19659	18837	3878	3838
IF39-1M	447906	27508	27103	26044	3485	3162
IF39-3M	299584	18635	18097	17440	5343	5043
IF39-6M	343561	21853	21344	20649	4713	4646
B4-1M	471386	29441	29151	28811	10129	9976
B4-3M	469119	28954	28565	27672	6961	6780
B4-6M	404434	25092	24811	24030	6786	6526
B40-1M	360691	22359	22115	21358	5675	5559
B40-3M	437408	27404	27087	26419	5926	5558
B40-6M	411491	26146	25745	24249	5352	4876
B45-1M	371419	23071	22692	21920	6523	6466
B45-3M	434493	27382	27101	26349	6078	5874
B45-6M	410950	26086	25746	24536	4038	3997
B46-1M	305698	18665	18481	13547	5113	3736
B46-3M	306518	19113	18821	17992	5816	5335
B46-6M	422388	27042	26789	26011	5984	5867
B47-1M	320566	19897	19597	18948	4609	4504
B47-3M	421788	25753	25481	24998	8070	7966
B47-6M	592974	37771	37415	36791	8261	8161
B48-1M	326815	20553	20227	19445	4947	3938
B49-1M	688972	42714	42551	41635	12039	11643
B49-3M	2490031	154536	153606	149886	45195	43127
B49-6M	414924	26293	25923	24848	6064	6023
B5-3M	362306	22270	21886	20696	4802	4710
B5-6M	515197	31366	30778	28859	6431	6289
IF6S-1M	370043	22702	22446	21664	6605	6450
IF6S-3M	356326	22139	21759	20768	5824	5617
IF6S-6M	386783	23870	23322	21380	5108	4935
IF6T-1M	424818	26221	25592	24067	5394	5142
IF6T-3M	310469	19136	18817	17861	4887	4743
IF6T-6M	463925	28178	27576	25643	6204	5978
B7-3M	396651	24688	24480	24057	6048	5986
B7-6M	364350	22163	21723	20660	4435	4285
M1-1	427511	26524	25618	24338	8172	7053
M1-3	456095	28897	27852	26075	9588	8061
M1-6	504662	31868	30434	27142	8013	6846
M12-1	560758	34921	33810	30458	7255	6662
M12-3	446551	28457	27744	25847	7465	6704
M12-6	441719	27553	26627	24419	8365	6999
M13-1	632800	39624	38399	36351	10347	9423
M13-3	435213	27233	26448	24949	7687	6659
M14-1	437041	27091	25572	22597	5324	4078
M14-3	407000	25313	24818	23726	5487	4796
M14-6	509268	31391	30050	27240	6722	4865
M16-1	426440	26367	24835	21862	5994	4937
M16-3	506425	31533	30746	29340	7920	7383

M16-6	430921	26691	25787	24533	6801	6204
M19-1	443119	27842	26928	24745	5604	4928
M19-3	620106	39197	38432	37150	8607	7532
M19-6	411947	25790	25242	23705	6113	5730
M2-3	296503	18377	17805	16758	5486	5060
M2-6	405129	24995	24452	23259	5989	5379
M20-3	526288	33751	32905	30686	7224	6555
M20-6	471487	29373	28426	26243	6420	5950
M28-1	617294	39146	37753	35170	10717	9463
M28-3	316258	19861	19037	17387	5579	4953
M28-6	530557	33171	32022	29560	9435	8408
M29-1	456456	28079	27200	25766	8033	6973
M29-3	310854	19540	18651	16891	6508	4844
M29-6	432053	26767	25746	23155	4869	3945
M3-1	518047	32198	31336	30354	11682	11355
M3-3	371316	23747	23203	22565	7279	7109
M3-6	570328	35591	34077	32386	11360	8290
M30-1	452394	28194	27480	26601	7683	7125
M30-3	352358	22079	21554	20445	5678	5572
M30-6	476959	29625	28902	27721	7754	7227
M33-1	443514	27475	26690	25706	7530	6976
M33-3	316035	19825	19107	18231	5671	5133
M33-6	476325	30395	29133	26409	7231	6518
M34-1	681021	41921	40801	39256	10189	7993
M34-3	452623	28191	27452	26397	6942	6054
M34-6	467867	28881	28077	26386	5975	5168
M35-1	487413	29670	28961	28013	8264	7536
M35-6	351351	22123	21497	20279	7053	5729
M37-1	466037	28625	27779	26115	8224	7424
M37-6	444218	27469	26403	24520	7835	5313
M39-1	483040	30175	28740	25862	6949	5736
M39-3	440609	27604	26837	25444	8164	7046
M39-6	363131	22675	21942	20821	6033	5266
M4-1	584122	36484	35398	33876	10153	9092
M4-3	387780	24312	23615	22638	7149	6706
M4-6	416937	26099	25231	23068	6837	5909
M40-1	464830	28476	27518	26165	10115	8451
M40-3	448153	27952	27132	25611	8510	7451
M40-6	454735	28388	27700	25977	9174	8082
M45-1	438173	27048	26376	25051	7413	6752
M45-3	355838	22261	21684	20472	7155	6151
M45-6	426805	26430	25698	24120	6323	5971
M46-1	457573	27971	26943	24089	5799	4951
M46-3	473312	29383	28547	26894	7226	6705
M46-6	466667	28750	27985	26924	7915	7345
M47-1	456985	28307	27620	26323	8267	7674

M47-3	576695	35810	35029	33317	9886	8659
M47-6	450641	28140	27156	25096	8014	6905
M48-1	727775	44708	43531	41723	12547	10616
M49-1	513139	31453	30625	29069	8363	7813
M49-3	638357	39701	38399	36593	12738	10378
M49-6	171399	10854	10473	9721	3515	3223
M5-1	541105	33626	32845	31497	8372	7815
M5-3	250698	16439	15985	15208	4387	4145
M5-6	399692	24884	24298	22922	6619	5956
M6-1	524837	32735	32091	30392	9242	8672
M6-3	406194	25487	24747	22896	7573	6685
M6-6	383436	24243	23677	22788	6674	6101
M7-1	529999	32737	32094	31084	7600	7271
M7-3	496964	31393	30794	29834	8575	8171
M7-6	431533	26811	25581	23189	6640	4771
PW1	421681	26533	25491	23441	7678	7199
PW12	423164	26333	25318	23584	8530	6528
PW13	455199	28529	27894	26445	7966	6836
PW14	511588	31656	30511	27519	7244	6378
PW16	494593	30376	29626	28061	8311	7562
PW19	441824	28087	27226	25781	7340	6741
PW2	454414	28477	27495	26064	8175	7010
PW20	290874	17587	17015	15928	4370	4086
PW28	338376	21143	20241	18525	5452	4898
PW29	389681	24169	23377	22472	6207	5810
PW3	483294	30171	28811	26979	8703	7657
PW30	383793	23703	23029	21871	5800	5575
PW33	417938	25843	24988	23928	6188	5936
PW34	627316	39143	38420	37475	8792	8200
PW35	468739	28704	27928	26691	8609	7594
PW37	445728	27909	27007	25279	7006	6242
PW39	507947	31392	30401	27688	8680	7278
PW4	460573	28513	27692	26411	8963	8319
PW40	552734	34502	33423	30795	8233	7528
PW45	529400	32892	31911	30281	9831	8953
PW46	458448	28926	27440	23959	7183	6479
PW47	402567	24801	24279	23005	6221	5842
PW48	323458	20476	19737	18157	6363	5506
PW49	470762	29360	28579	27304	8375	7784
PW5	479790	29711	28906	27311	7120	5972
PW6	454225	28440	27505	25199	8517	7633
PW7	692479	42893	40957	36344	9276	7946
average	**451900**	**28120**	**27446**	**26002**	**7326**	**6659**

ST2. Kruskal-Wallis pairwise comparisons for Evenness, alpha diversity.

Group 1	Group 2	H	p-value	q-value
IF1 (n=20)	IF3 (n=25)	0,00052	0,98	0,98
IF1 (n=20)	IF6 (n=23)	6,41	0,011	0,017
IF1 (n=20)	M1 (n=25)	27,60	1,49E-07	3,48E-07
IF1 (n=20)	M3 (n=24)	27,88	1,29E-07	3,40E-07
IF1 (n=20)	M6 (n=25)	30,05	4,21E-08	1,47E-07
IF1 (n=20)	PW (n=27)	32,51	1,18E-08	5,66E-08
IF3 (n=25)	IF6 (n=23)	8,41	3,74E-03	6,04E-03
IF3 (n=25)	M1 (n=25)	32,87	9,84E-09	5,66E-08
IF3 (n=25)	M3 (n=24)	32,26	1,35E-08	5,66E-08
IF3 (n=25)	M6 (n=25)	33,77	6,20E-09	5,66E-08
IF3 (n=25)	PW (n=27)	37,98	7,14E-10	1,50E-08
IF6 (n=23)	M1 (n=25)	23,62	1,17E-06	2,14E-06
IF6 (n=23)	M3 (n=24)	23,54	1,22E-06	2,14E-06
IF6 (n=23)	M6 (n=25)	26,09	3,26E-07	6,84E-07
IF6 (n=23)	PW (n=27)	29,18	6,60E-08	1,98E-07
M1 (n=25)	M3 (n=24)	0,44	0,51	0,63
M1 (n=25)	M6 (n=25)	0,10	0,75	0,83
M1 (n=25)	PW (n=27)	0,014	0,91	0,95
M3 (n=24)	M6 (n=25)	0,61	0,44	0,57
M3 (n=24)	PW (n=27)	1,24	0,27	0,37
M6 (n=25)	PW (n=27)	0,33	0,56	0,66

ST3. Kruskal-Wallis pairwise comparisons for Faith's phylogenetic diversity, alpha diversity.

Group 1	Group 2	H	p-value	q-value
IF1 (n=20)	IF3 (n=25)	2,63	0,10	0,15
IF1 (n=20)	IF6 (n=23)	14,43	0,00015	0,00024
IF1 (n=20)	M1 (n=25)	32,61	1,13E-08	2,15E-08
IF1 (n=20)	M3 (n=24)	32,00	1,54E-08	2,70E-08
IF1 (n=20)	M6 (n=25)	32,61	1,13E-08	2,15E-08
IF1 (n=20)	PW (n=27)	33,75	6,27E-09	1,46E-08
IF3 (n=25)	IF6 (n=23)	4,39	3,62E-02	5,43E-02
IF3 (n=25)	M1 (n=25)	36,76	1,33E-09	7,89E-09
IF3 (n=25)	M3 (n=24)	36,00	1,97E-09	8,29E-09
IF3 (n=25)	M6 (n=25)	36,76	1,33E-09	7,89E-09
IF3 (n=25)	PW (n=27)	38,21	6,36E-10	7,89E-09
IF6 (n=23)	M1 (n=25)	35,20	2,97E-09	8,91E-09
IF6 (n=23)	M3 (n=24)	34,50	4,26E-09	1,12E-08
IF6 (n=23)	M6 (n=25)	35,20	2,97E-09	8,91E-09
IF6 (n=23)	PW (n=27)	36,53	1,50E-09	7,89E-09
M1 (n=25)	M3 (n=24)	0,19	0,66	0,69
M1 (n=25)	M6 (n=25)	0,45	0,50	0,56
M1 (n=25)	PW (n=27)	2,45	0,12	0,15
M3 (n=24)	M6 (n=25)	0,014	0,90	0,90
M3 (n=24)	PW (n=27)	0,96	0,33	0,38
M6 (n=25)	PW (n=27)	1,62	0,20	0,25

ST4. Faith's phylogenetic diversity within a Linear Mixed Effected (LME) model, alpha diversity.

	Coef.	**Std.Err.**	**z**	**P>|z|**	**[0.025**	**0.975]**
Intercept	3,06	0,26	11,64	0	2,55	3,58
Groups [T.M]	5,12	0,27	19,07	0	4,59	5,65
Groups [T.P]	5,98	0,38	15,92	0	5,24	6,72
time	0,11	0,052	2,10	0,036	0,007	0,21
Group Var	0,37	0,21				

ST5. Pairwise permanova results for unweighted UniFrac distance, beta diversity.

Group 1	Group 2	Sample size	Permutations	pseudo-F	p-value	q-value
IF1	IF3	45	999	2,26	0,007	0,0098
IF1	IF6	43	999	7,87	0,001	0,0015
IF1	M1	45	999	31,54	0,001	0,0015
IF1	M3	44	999	30,16	0,001	0,0015
IF1	M6	45	999	31,24	0,001	0,0015
IF1	PW	47	999	34,82	0,001	0,0015
IF3	IF6	48	999	3,75	0,001	0,0015
IF3	M1	50	999	26,56	0,001	0,0015
IF3	M3	49	999	26,07	0,001	0,0015
IF3	M6	50	999	26,55	0,001	0,0015
IF3	PW	52	999	30,54	0,001	0,0015
IF6	M1	48	999	24,26	0,001	0,0015
IF6	M3	47	999	24,46	0,001	0,0015
IF6	M6	48	999	24,63	0,001	0,0015
IF6	PW	50	999	29,37	0,001	0,0015
M1	M3	49	999	0,95	0,40	0,46
M1	M6	50	999	0,55	0,92	0,92
M1	PW	52	999	1,97	0,05	0,066
M3	M6	49	999	0,60	0,83	0,87
M3	PW	51	999	0,80	0,55	0,61
M6	PW	52	999	1,42	0,14	0,17

ST6. Pairwise permanova results for weighted UniFrac distance, beta diversity.

Group 1	Group 2	Sample size	Permutations	pseudo-F	p-value	q-value
IF1	IF3	45	999	1,17	0,32	0,33
IF1	IF6	43	999	9,89	0,001	0,0015
IF1	M1	45	999	97,34	0,001	0,0015
IF1	M3	44	999	100,15	0,001	0,0015
IF1	M6	45	999	115,76	0,001	0,0015
IF1	PW	47	999	122,57	0,001	0,0015
IF3	IF6	48	999	8,72	0,001	0,0015
IF3	M1	50	999	101,30	0,001	0,0015
IF3	M3	49	999	105,74	0,001	0,0015
IF3	M6	50	999	122,29	0,001	0,0015
IF3	PW	52	999	126,79	0,001	0,0015
IF6	M1	48	999	43,04	0,001	0,0015
IF6	M3	47	999	47,38	0,001	0,0015
IF6	M6	48	999	55,97	0,001	0,0015
IF6	PW	50	999	56,08	0,001	0,0015
M1	M3	49	999	1,53	0,16	0,21
M1	M6	50	999	2,01	0,053	0,074
M1	PW	52	999	1,40	0,18	0,22
M3	M6	49	999	0,85	0,52	0,52
M3	PW	51	999	1,37	0,20	0,23
M6	PW	52	999	1,35	0,21	0,23

Apêndice IV: Material Suplementar Capítulo 2.

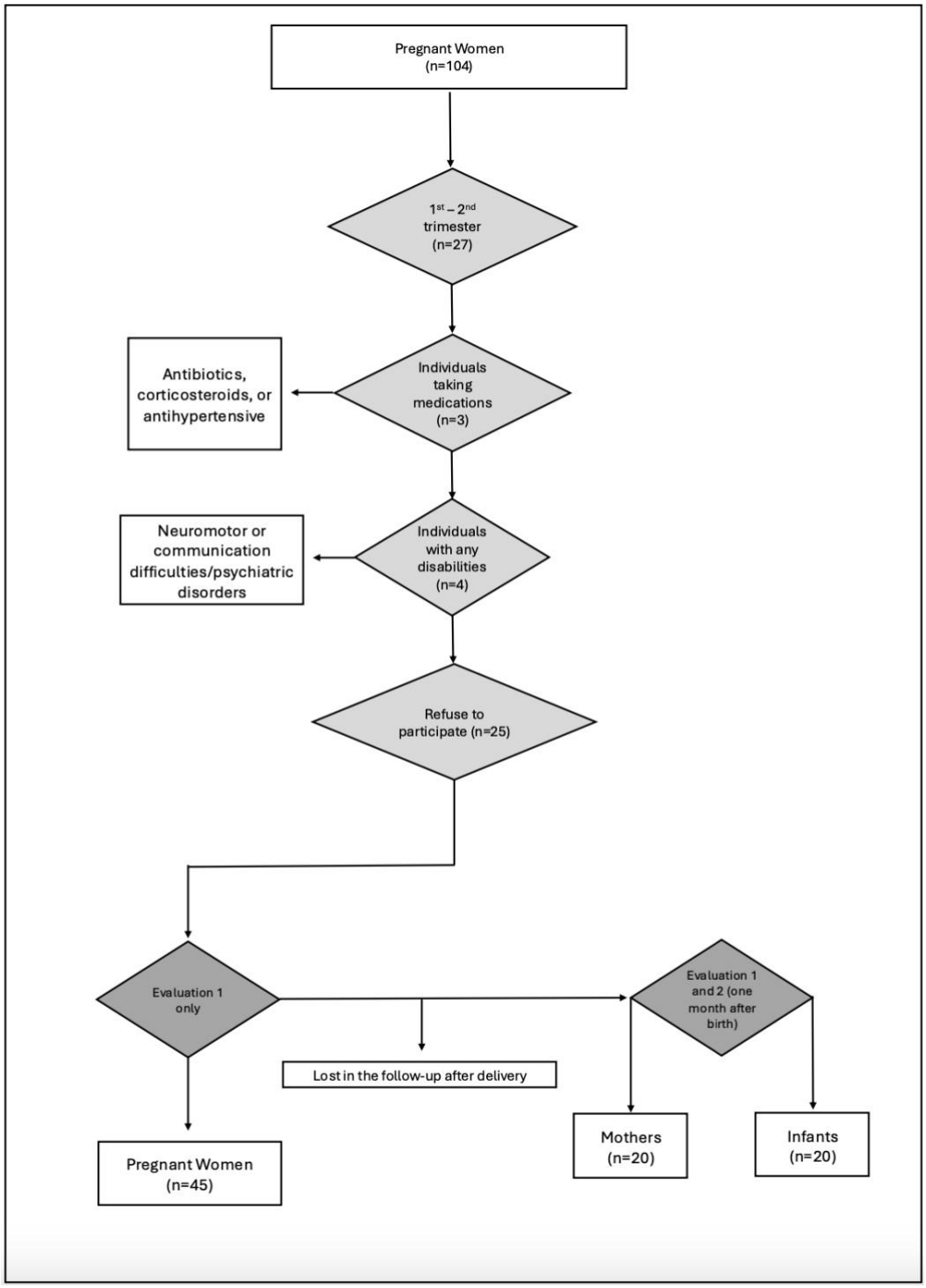


Figure S1. Flow chart of the sample selection.

Table S1 - Supplementary Table 1

Table S1. Details of the proteins identified in the study, including UniProt entry terms, oficial protein names, gene symbols, and their respective experimental groups.

UniProt access number	Entry Names	Protein Names	Gene symbols	Groups
B4DXF1	B4DXF1_HUMAN	3'-phosphate/5'-hydroxy nucleic acid ligase	RTCB	PP
Q8TE59	ATS19_HUMAN	A disintegrin and metalloproteinase with thrombospondin motifs 19	ADAMTS19	IN
Q6UY14	ATL4_HUMAN	ADAMTS-like protein 4	ADAMTSL4	IN
P02768	ALBU_HUMAN	Albumin	ALB	PP IN
P0DUB6	AMY1A_HUMAN	Alpha-amylase 1A	AMY1A	PW PP IN
P0DTE7	AMY1B_HUMAN	Alpha-amylase 1B	AMY1B	PW PP IN
P0DTE8	AMY1C_HUMAN	Alpha-amylase 1C	AMY1C	PW PP IN
P19961	AMY2B_HUMAN	Alpha-amylase 2B	AMY2B	PW PP IN
P54920	SNAA_HUMAN	Alpha-soluble NSF attachment protein	NAPA	PW
P04083	ANXA1_HUMAN	Annexin A1	ANXA1	PP
P54136	SYRC_HUMAN	Arginine--tRNA ligase	RARS1	IN

P17858	PFKAL_HUMAN	ATP-dependent 6-phosphofructokinase, liver type	PFKL			IN
P60709	ACTB_HUMAN	Beta-actin	ACTB		PP	IN
A2RUR9	C144A_HUMAN	CCDC144C-like coiled-coil domain-containing protein	CCDC144A			IN
A0AAG2SVG9	A0AAG2SVG9_HUMAN	CCDC144C-like coiled-coil domain-containing protein	LOC100288966		PP	
Q13740	CD166_HUMAN	CD166 antigen	ALCAM		PP	
P10645	CMGA_HUMAN	Chromogranin-A	CHGA			IN
Q14050	CO9A3_HUMAN	Collagen alpha-3(IX) chain	COL9A3			IN
P01036	CYTS_HUMAN	Cystatin-S	CST4		PP	
Q9BXX0	EMIL2_HUMAN	EMILIN-2	EMILIN2	PW		
Q96CU9	FXRD1_HUMAN	FAD-dependent oxidoreductase	FOXRED1			IN
I3L401	I3L401_HUMAN	FCP1 homology domain-containing protein	I3L401		PP	
P35555	FBN1_HUMAN	Fibrillin-1	FBN1	PW		
Q75N90	FBN3_HUMAN	Fibrillin-3	FBN3			IN
P49915	GUAA_HUMAN	GMP synthase	GMPS		PP	
A0A140TA62	A0A140TA62_HUMAN	IF rod domain-containing protein	LOC100653049		PP	
Q6P089	Q6P089_HUMAN	IGH@ protein	IGH@	PW	PP	

Q0KKI6	Q0KKI6_HUMAN	Immunoblobulin light chain (Fragment)	Q0KKI6	PW		
P0DOX2	IGA2_HUMAN	Immunoglobulin alpha-2 heavy chain	IgH		PP	
Q9NPP6	Q9NPP6_HUMAN	Immunoglobulin heavy chain variant	IGH	PW	PP	
P01876	IGHA1_HUMAN	Immunoglobulin heavy constant alpha 1	IGHA1	PW	PP	
P01877	IGHA2_HUMAN	Immunoglobulin heavy constant alpha 2	IGHA2	PW	PP	
P13645	K1C10_HUMAN	Keratin, type I cytoskeletal 10	KRT10	PW		
Q9C075	K1C23_HUMAN	Keratin, type I cytoskeletal 23	KRT23		PP	
P61626	LYSC_HUMAN	Lysozyme C	LYZ		PP	I N
Q8N119	MMP21_HUMAN	Matrix metalloproteinase-21	MMP21			I N
Q9HC84	MUC5B_HUMAN	Mucin-5B	MUC5B	PW		
P59665	DEF1_HUMAN	Neutrophil defensin 1	DEFA1	PW		
P59666	DEF3_HUMAN	Neutrophil defensin 3	DEFA3	PW		
P14543	NID1_HUMAN	Nidogen-1	NID1			I N
Q8N323	NXPE1_HUMAN	NXPE family member 1	NXPE1		PP	
P04746	AMYP_HUMAN	Pancreatic alpha-amylase	AMY2A	PW	PP	I N
Q92626	PXDN_HUMAN	Peroxidasin homolog	PXDN	PW		
P14555	PA2GA_HUMAN	Phospholipase A2 (Phospholipase A2, membrane associated)	PLA2G2A			I N

P01833	PIGR_HUMAN	Polymeric immunoglobulin receptor	PIGR			I N
P49792	RBP2_HUMAN	RanBP2-type domain-containing protein	RANBP2			I N
Q8WY24	Q8WY24_HUMAN	SNC66 protein	SNC66	PW	PP	
Q9UP60	Q9UP60_HUMAN	SNC73 protein	SNC73	PW		
Q92673	SORL_HUMAN	Sortilin-related receptor	SORL1		PP	
O43897	TLL1_HUMAN	Tolloid-like protein 1	TLL1			I N
Q9NVV0	TM38B_HUMAN	Trimeric intracellular cation channel family protein	TMEM38B			I N
Q96DK0	Q96DK0_HUMAN	Tro alpha1 H,myeloma - cDNA FLJ41981	Q96DK0	PW		
Q6ZVX0	Q6ZVX0_HUMAN	Tro alpha1 H,myeloma - cDNA FLJ25298I	Q6ZVX0		PP	
Q9Y5K8	VATD_HUMAN	V-type proton ATPase subunit D	ATP6V1D			I N
Q8N972	ZN709_HUMAN	Zinc finger protein 709	ZNF709		PP	