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ORIGINAL ARTICLE

Microbial profile of placentas from Tanzanian mothers with adverse pregnancy outcomes and periodontitis

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Abstract

Aim: To investigate microbial profiles in placentas from a population of East African mothers with and without adverse pregnancy outcomes and with regard to their periodontal status.

Material and Methods: Thirty-six placentas from pregnant women from Tanzania were classified into three groups according to both pregnancy outcome and the mother's periodontal health. The microbial composition in each group was then compared using 16S rRNA metagenomics. Additionally, placenta specimens were analyzed histologically for chorioamnionitis by a single pathologist blinded to the clinical data.

Results: The greatest differences were observed in the group of mothers with periodontitis. The microbial load was low in all three groups of mothers. Periodontitis had a notable influence on the structure of the placental microbiota. Three phyla and 44 genera were associated with periodontitis, whereas only the *Tenericutes* phylum was associated with the adverse pregnancy variable. *Streptococcaceae* and *Mycoplasmataceae* families were associated with both periodontitis and adverse pregnancy outcomes. Finally, although the differences for chorioamnionitis were not significant, this intra-amniotic infection was more frequent in the placentas from mothers with periodontitis.

Conclusions: Our findings suggest that bacteria from the oral cavity may involve the feto-placental unit, and that periodontitis may be a modulating factor of the microbial community present in this niche.

KEYWORDS
chorioamnionitis, maternal periodontitis, obstetric complications, periodontal disease, placental microbiome, prematurity

1 | INTRODUCTION

Until recently, the placenta was widely believed to be a sterile element serving as a barrier against harmful agents. It provides oxygen and nutrients and is a promoter of the exchange of molecules between mother and fetus. Normally, the presence of bacteria in the placenta or the amniotic fluid has been associated with infections produced by microorganisms invading from the lower genital tract (Onderdonk et al., 2016). However, with the use of new sequencing technologies, some authors have reported that low-abundance microbiota can be detected in the placenta of healthy mothers of full-term deliveries (Aagaard et al., 2014). Although the origin of this microbiota is still under discussion, it is more similar to the pregnant mother's oral microbiome than to her gut microbiome (Aagaard et al., 2014; Gomez-Arango et al., 2017). Furthermore, this low-abundance placental microbiota does not appear to comprise pathogenic bacteria, and it has been suggested that it contributes to the normal development of the fetal immune system (Tlaskalová-Hogenová et al., 2004; Van Belkum et al., 2020). Prior to the use of these sequencing techniques, the presence of microorganisms in the placenta was always considered a risk factor for adverse pregnancy outcomes, suggesting that pathogenic bacteria colonize the placenta by hematogenous route or from the vagina. These bacteria are believed to cause intrauterine inflammation and infection, leading to preterm labor, preterm birth (PTB), and preterm brain injury (Malaeb & Dammann, 2009; Tomlinson et al., 2019). PTB is a major cause of neonatal mortality worldwide, and it has been estimated that between 25% and 40% of spontaneous PTB are caused by intrauterine infections (Goldenberg et al., 2008), which are thought to provoke an inflammatory response in the mother and/or fetus, inducing early labor and membrane rupture (Sykes et al., 2012). Moreover, some studies have associated the presence of periodontal pathogens in the placenta and/or amniotic fluid with PTB. In a case–control study, Offenbacher et al., (1996) concluded that there was a relationship between periodontitis and PTB and/or low birth weight (LBW) (Offenbacher et al., 1996). Since that ground-breaking work, a series of studies have been carried out, and their results published in meta-analyses and reviews. In 2007, Vergnes and Sixou described that periodontitis has a risk factor odds ratio of 2.83 for preterm LBW, and in their meta-analysis supported the concept of periodontitis as a risk factor for PTB (Vergnes & Sixou, 2007). In an earlier cross-sectional study by our group, a logistic regression was performed and adjusted odds ratio (aOR) values were obtained between adverse pregnancy outcomes and maternal periodontal disease. The authors concluded that periodontitis is a potential independent risk indicator for pre-eclampsia (aOR=4.12), low birth weight (aOR=2.41), and preterm birth (aOR=2.32) (Gesase et al., 2018). In addition, the non-invasive nature of periodontal therapy may bring important benefits in these cases (Jajoo et al., 2020; Vergnes & Sixou, 2007).

Furthermore, several animal model studies have shown that subcutaneous infections caused by periodontal pathogens such as *Porphyromonas gingivalis*, or putative periodontal pathogens such as *Campylobacter rectus* or *Fusobacterium nucleatum*, can affect the development and viability of mouse fetuses (Stockham et al., 2015; Udagawa et al., 2018; Yeo et al., 2005). Likewise, periodontal disease-related oral bacteria have been detected in the placental tissues or amniotic fluid of mothers with and without adverse pregnancy outcomes, although their presence is significantly more frequent in cases of PTB and LBW (Blanc et al., 2015; Ye et al., 2020).

Based on the clinical and histopathological results of our previous study in a similar context, we hypothesized that placentas from mothers with active periodontitis would present characteristic microbiological profiles, which would be related to adverse pregnancy outcomes (Gesase et al., 2018). In another study in the same population, a subgroup of pregnant women with periodontitis presented statistically significant associations with high grades or stages of chorioamnionitis and lower feto-placental ratio (Nadal et al., 2019).

Using 16S rRNA sequencing, in the present study, we examined microbial profile and abundance in placentas from mothers with and without adverse pregnancy outcomes and in relation to their periodontal health status.

2 | MATERIAL AND METHODS

2.1 | Study subjects and sample collection

From the study carried out by Gesase et al., (2018), we included all pregnant women (N=1117) delivering at the Kilimanjaro Christian Medical Centre—KCMC—Moshi (Tanzania), aged from 18 to 46 years, with singleton intrauterine fetuses of gestational age between 28 and 42 weeks. We excluded multiple gestations and women with any systemic infection. Socio-demographic characteristics, previous obstetric history, and information on the index pregnancy were recorded. Periodontal examination was performed by a single trained examiner 24h after delivery. We used the criteria of periodontal

disease defined by Gomes-Filho et al., (2007), according to the four types of exposure measurement (EM). Based on their results, in our study, we took the values of EM3 and EM4 to indicate periodontitis. This clinical index highlights the importance of registering bleeding on probing in deep active periodontal pockets (Moncunill-Mira et al., 2021).

Adverse maternal and/or fetal outcomes were recorded in 9.8% of the sample (n=110). The prevalence of periodontal disease in this large sample was 14.2% (n=159). Nevertheless, maternal periodontitis was diagnosed in 28.2% of the women who presented adverse maternal and/or fetal outcomes, but in only 12.7% of the women without these complications. In that clinical study, all pregnant women with any other infection or disease were excluded, and the statistical analysis assessed the factors that presented significant differences between the groups studied and might have influenced these adverse outcomes rates. Periodontal disease was significantly associated with higher odds of pre-eclampsia; low birth weight and preterm birth.

2.2 | Tissue harvesting, sample preparation, and histopathological study

In the study carried out by Gesase et al., 2018, placentas were aseptically collected immediately after delivery. A trained obstetrician used a sterile technique to harvest the samples consistently after dissecting the membranes and taking a 2x2 cm segment from the inside of the placental disk around the paracentral section. Specimens were immediately fixed in 10% buffered formalin, and after 48h, they were embedded aseptically in paraffin. This is a standard technique used in clinical and research laboratories to create a formalin-fixed paraffin-embedded (FFPE) block of tissue.

After discarding some paraffin-embedded placenta samples with inadequate sealing or packaging and limited histological material, suitable samples (twelve for each group) were selected at random from 36 women who, during their pregnancy, either: i) n=12 presented with periodontitis and adverse pregnancy outcomes (PA); ii) n=12 did not present periodontitis during pregnancy but suffered adverse pregnancy outcomes (NPA); or iii) n=12 did not suffer periodontitis and whose pregnancy was carried to term (NPNA).

2.3 | DNA extraction

Paraffin-embedded tissue specimens were aseptically handled and cut into 10-micron-thick sections using a microtome (Microm HM325; Thermo Fisher Scientific Inc., Waltham, MA, USA) following the protocol described by Blanc et al., 2015. The slides of each block were stored in sterile tubes at 4°C until they were deparaffinized with Xylol. Genomic DNA extractions from 25 mg of the placenta slides were performed using a QIAamp DNA FFPE Tissue Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. Afterward, DNA concentration was quantified using a Nanodrop ND-1000 UV-Vis spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA). Two samples from paraffin blocks without placental tissue were also processed and sequenced as negative controls. Prior to the next-generation sequencing (NGS) library preparation, microbial DNA was enriched by removing the methylated host DNA. To this end, the NEBNext Microbiome DNA Enrichment Kit (New England Biolabs Inc.) was applied to the purified genomic DNA following the manufacturer's instructions. Enriched DNA concentrations were quantified with Qubit 4.0 fluorometer (Thermo Fisher Scientific). Researchers carried out all techniques and procedures using gloves and surgical masks.

2.4 | 16S rDNA amplicon sequencing

The hypervariable V3–V4 region of the 16S rRNA gene was amplified to taxonomically characterize the microbial composition of the samples. Dual indices were attached to the amplified DNA using the Nextera XT Index kit (Illumina), and the resulting amplicons were quantified using a Qubit 4.0 fluorometer. The libraries were then pooled and paired-end sequenced by a MiSeq platform (Illumina) using a 500-cycle MiSeq Reagent kit v2. Sequencing was carried out in the Servei de Genòmica i Bioinformàtica [*Genomics and Bioinformatics Service*] at the Universitat Autònoma de Barcelona.

2.5 | Pathology

Additionally, placenta specimens were analyzed histologically for chorioamnionitis by a single pathologist blinded to the clinical data. Fetal and maternal responses, when present, were grouped according to the classification of the Amsterdam Placental Workshop Group Consensus (Khong et al., 2016).

2.6 | Statistical analysis

Clinical data were evaluated using R version 4.0.2. Descriptive analysis was calculated as mean ±standard deviation or as percentage of cases, depending on the type of variable. Pearson's chi-squared, Kruskal–Wallis rank sum, and ANOVA tests were used to assess the statistical significance of the differences between the groups, setting the level of significance at alpha =0.05. Before applying the ANOVA test, the normality and homoscedasticity of the data were assessed with the Shapiro–Wilk normality test and Bartlett's test, respectively. Moreover, Tukey and Dunn post hoc tests were performed to confirm the significant comparisons after ANOVA and Kruskal–Wallis rank sum tests, respectively.

2.7 | Bioinformatics analysis

The demultiplexed raw data were downloaded via ftp from the Servei de Genòmica i Bioinformàtica at the Universitat Autònoma

de Barcelona. The reads were trimmed by Phred Score >20 and assembled using FLASH software (Magoč & Salzberg, 2011). The next steps were performed using the Mothur v1.44.3 suite of analysis (Schloss et al., 2009). First, any remaining barcode or primer was removed from the reads. Then, the reads with a length between 400 and 480 nt were retained. Chimera removal was accomplished by the Uchime algorithm (Edgar et al., 2011) using the SILVA set of 16S sequences (release 128) as reference (Quast et al., 2013). All samples were normalized by randomly subsetting 21,000 sequences per sample, and further analysis was carried out with this rarefied dataset. The taxonomic assessment was performed using the ribosomal database project (RDP) classifier v2.12 (Wang et al., 2007). The operational taxonomic unit (OTU)-picking approach was performed with the UCLUST algorithm (using a 97% identity threshold) implemented in the USEARCH v8.0.1623 software (Edgar, 2010), and discarding singletons and doubletons during the clustering into OTUs. Differences between different groups and conditions, as well as interactions between variables, were statistically tested by linear mixed model (LMM) approaches on log-transformed data using the R (v 4.0) packages nlme, r2glmm and MuMIn (http://cran.r-project.org), and taxonomic categories with differential distribution across groups were selected based on *p*-values ≤0.01. Pairwise comparison of selected taxonomy categories with differential abundance was performed with the Mann–Whitney U test for independent samples, and *p*-values were corrected with the Benjamini and Hochberg method. Taxonomy identity of OTUs with differential abundance was assessed using a BLAST-based approach with the NCBI non-redundant 16S database, as well as the SINA aligner with a SILVA database annotation (Pruesse et al., 2012). Alpha diversity was assessed with common descriptors (Chao's index, Observed richness, Shannon's index, and reciprocal Simpson's index) through the QIIME v1.9.1 suite of analysis (Caporaso et al., 2010). Then, the statistical assessment of individual parameters and comparison between groups was done with Kruskal–Wallis or ANOVA statistical tests (according to Shapiro–Wilk normality test results). Both the alpha diversity and the statistical analysis were run in R http://cran.r-project.org). QIIME was also used to analyze the global structure of microbial communities (beta diversity) through weighted and unweighted UniFrac metrics, as well as to perform principal coordinate analysis (PcoA) and statistical assessment using a permutation-based test (PERMANOVA) (Caporaso et al., 2010). Graphics and plots were designed in R and ggplot2 package (http://cran.r-project.org).

3 | RESULTS

3.1 | Demographic characteristics of the subjects

The clinical and demographic data of the mothers studied are shown in Table 1. The comparison of the three groups did not reveal significant differences for the following variables: age, marital status, employment status, placental weight, or blood pressure. For this last variable, the mean value in the NPNA group was lower than the values presented by the other two groups, but no significant differences were observed. No significant differences were observed between the two groups of mothers who presented with adverse pregnancy outcomes (PA and NPA), for any of the clinical variables analyzed. However, fetal weight was lower in the group of mothers with periodontitis (PA), even though their gestational age was higher (34.25 vs. 33.5). When the two groups were compared with the NPNA group, significant differences were found for all the clinical variables evaluated except placental weight and arterial hypertension.

3.2 | Data denoizing

An initial assessment aiming to determine the potential level of contamination of these very low bacterial load samples was achieved by performing multivariate analysis of OTUs predicted to be present in the whole dataset analyzed. Using weighted UniFrac metrics, which simultaneously estimates phylogenetics, occurrence, and abundance profiles in OTUs, followed by a PCoA, we were able to distinguish samples and groups according to the whole microbial structure (Figure 1). This analysis indicated that some samples, particularly from the PA group, seem to be strongly influenced by the background noise of DNA present in negative controls, suggesting the existence of a very low bacterial DNA load. Consequently, we corrected all the taxonomy proportions according to those observed in the negative controls in order to minimize the noise produced by contaminant DNA present in the reagents and the procedures followed for 16S amplicon sequencing, which provides more accurate microbiome information.

3.3 | Beta diversity

The composition and structure of the microbial community as a whole were assessed by using the OTU information obtained from clustering approaches at 97% sequence identity and representing the phylotype distribution across samples and groups. All the above information was compiled in a multivariate analysis based on weighted UniFrac metrics followed by a PCoA evaluation. We made comparisons at different levels involving coupled grouping criteria (periodontitis status +adverse pregnancy) and individual criteria. A permutation-based test (Permanova) was used to assess the differential distribution of species and their abundances among groups. The multivariate analysis and pseudo-F test indicated that the microbial structure of the samples differs between groups (Figure 2A). In the PCoA plot, the distribution of samples by group (NPNA, NPA, and PA) is well distinguished, where PC1 (principal coordinate 1) discriminates PA from NPNA and NPA and PC2 discriminates the NPNA group from NPA and PA counterparts.

Interestingly, when we explored the individual parameters, we observed that periodontitis status was the more discriminant variable of the two variables combined (Figure 2B and 2C).

TABLE 1 Description and comparison of the socio-demographic and clinical variables between the three groups of pregnant women studied

Variable	PA (n=12)	NPA (n=12)	NPNA (n=12)	*p*-value
Age (years)	29.33 ± 5.47	28.75 ± 6.50	31.42 ± 3.80	0.45[d]
Marital status (% married)	91.67	91.67	100.00	0.589[a]
Employed (% yes)	25	50	25	0.325[a]
Adversity type (%)				
Eclampsia	16.67	-	-	
PPROM	8.33	8.33	-	
PPROM/Corio	-	8.33	-	
Preclampsia	50,00	50,00	-	
Preclampsia Pret Stillbirth	-	8.33	-	
Preterm	25.00	25.00	-	
Gestational age (weeks)	34.25 ± 3.77	33.5 ± 4.4	39.17 ± 1.59	0.0006695[b]
				0.40[c,φ]
				0.0007[c,ρ]
				0.0003[c,Φ]
Fetus weight (gr)	2037.5 ± 671.88[φ,ρ]	2108.33 ± 840.41[φ,Φ]	3375 ± 541.25[ρ,Φ]	3.841E−05[d]
				0.966[e,φ]
				0.00012[e,ρ]
				0.00026[e,Φ]
Placenta weight (gr)	377.5 ± 126.78	355.5 ± 132.868	437.5 ± 118.94	0.272[d]
Cord diameter (cm)	1.4 ± 0.36	1.73 ± 0.26	2.7 ± 0.71	0.00035[b]
				0.056[c,φ]
				0.00[c,ρ]
				0.0088[c,Φ]
Arterial Hypertension (%)	58.33	41.67	16.67	0.1085[a]
Gravidity	2.17 ± 1.70	2.17 ± 0.72	3.25 ± 0.97	0.0062[b]
				0.2801[c,φ]
				0.0013[c,ρ]
				0.0077[c,Φ]
Parity	1.5 ± 1.17	1.42 ± 0.67	2.25 ± 0.97	0.025[b]
				0.4106[c,φ]
				0.0071[c,ρ]
				0.0088[c,Φ]
Live	1.42 ± 1.16	1.25 ± 0.87	2.08 ± 0.79	0.0203[b]
				0.4662[c,φ]
				0.0070[c,ρ]
				0.0088[c,Φ]

Abbreviation: PA: periodontitis and adverse pregnancy. NPA: non-periodontitis and adverse pregnancy. NPNA: non-periodontitis and non-adverse pregnancy.

[a]Chi-square test.

[b]Kruskal–Wallis test.

[c]Dunn's test.

[d]ANOVA.

[e]Tukey's test.

φ= PA *vs* NPA.

ρ= PA *vs* NPNA.

Φ= NPA *vs* NPNA.

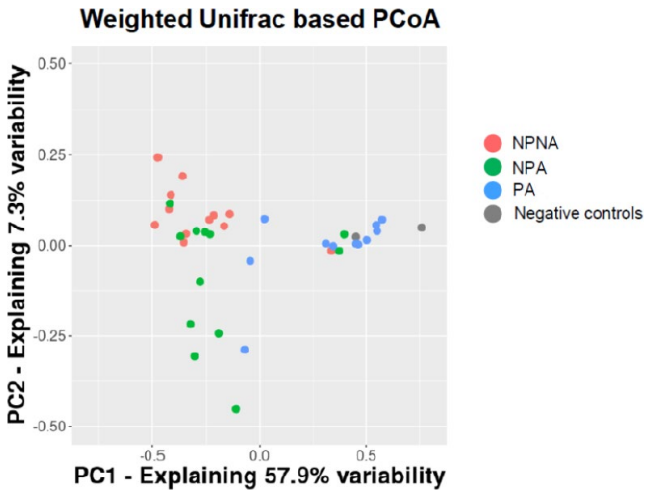

FIGURE 1 Multivariate PCoA analysis based on weighted UniFrac distances of the entire set of samples included in the study to determine noise and potential contamination in samples

3.4 | Differential abundance in taxonomy categories

We performed a hierarchical assessment to detect distinctive features in different groups evaluated at phylum, family, and genus levels. At phylum level, we discerned a total of five categories, although only four were differentially present in samples, depending on periodontitis and adverse pregnancy status (Figure 3). Three of these phyla—*Proteobacteria*, *Bacteroidetes*, and *Actinobacteria*—were associated with periodontitis status, whereas *Tenericutes* phylum was associated with the adverse pregnancy variable. *Proteobacteria* and *Actinobacteria* phyla were notably associated with samples where periodontitis was present. Conversely, *Bacteroidetes* phylum were more predominant in samples without periodontitis. Moreover, the *Tenericutes* phylum was strongly associated with placental samples of adverse pregnancy.

At the family level, we observed differential abundance, almost exclusively associated with periodontitis status (Table 2). Twenty-seven bacterial families, belonging mainly to the *Proteobacteria*, *Firmicutes*, and *Actinobacteria* phyla, were found to be positively associated with periodontitis. Conversely, four families, each one belonging to a different phylum, were negatively associated with the periodontitis variable: *Ruminococcaceae (Firmicutes)*, *Porphyromonadaceae (Bacteroidetes)*, *Rhodospirillaceae (Proteobacteria)*, and *Anaeroplasmataceae (Tenericutes)*. If we study the data regarding the adverse pregnancy outcomes, we find three families associated with this condition, two of them—*Streptococcaceae* and *Mycoplasmataceae*—also associated with periodontitis. Only the *Anaeroplasmataceae* family was negatively associated with periodontitis and positively associated with adverse pregnancy.

Forty-four genera were significantly associated with the periodontitis variable, and only five were negatively associated with this disease. The *Holdemanella*, *Mycoplasma*, *Streptococcus*, and *Anaerostipes* genera were associated with adverse pregnancy, and the first three being also associated with periodontitis. The *Slackia*, *Paraeggerthella*, and *Oxalobacter* genera showed a negative association with adverse pregnancy (Table 3).

3.5 | Pathology

Chorioamnionitis was identified in 10 out of the 36 cases, four of them in the PA group. These four cases were either stage 2 (2) or grade 2 (2) for maternal response, with one additionally showing stage 2 grade 1 fetal response. This fetal response was only seen in one case belonging to the PA group. All in all, although the differences for chorioamnionitis were not significant, this intra-amniotic infection was more frequent in the placentas from mothers with periodontitis.

4 | DISCUSSION

Preterm birth (PTB) and low birth weight (LBW) are the leading causes of neonatal death and infant mortality. Various risk factors have been described, including socioeconomic status, the age of the mothers (over 37 and under 17), diabetes mellitus, alcohol and tobacco use during pregnancy, high blood pressure, inadequate prenatal care, genitourinary infections, among others (Chang et al., 2013; Jajoo et al., 2020). For many years, intrauterine infections have also been associated with PTB. It is postulated that these infections could have an ascending origin, with pathogens ascending from the vaginal region toward the placenta and invading the membranes of the uterine cavity; alternatively, they may travel via the hematogenous route from distant locations such as the mouth. Several case-control studies have confirmed that periodontitis may increase the risk of PTB or LBW (Goldenberg et al., 2008; Jajoo et al., 2020; Offenbacher et al., 1996). Two pathogenic mechanisms have been proposed to explain the association between periodontitis and adverse pregnancy outcomes: i) a direct pathway, in which certain oral pathogens from the periodontal biofilm such as Gram-negative anaerobic bacteria invade the feto-placental unit, causing a local inflammation that may trigger an adverse outcome; ii) an indirect pathway, in which pro-inflammatory cytokines are produced locally in the periodontium and then reach the placenta or liver via the bloodstream, where other types of inflammatory cytokines or acute-phase reactants are generated and trigger adverse pregnancy outcomes (Blanc V. et al., 2015). Subsequently, a series of studies using molecular tools (PCR) detected the presence of periodontopathogens in amniotic fluid and in the placentas of women who presented with adverse maternal outcome, including *Porphyromonas gingivalis*, *Tannerella forsythia*, *Treponema denticola*, *Campilobacter rectus*, *Fusobacterium nucleatum*, and *Aggregatibacter actinomycetemcomitans* (Blanc et al., 2015; Chaparro et al., 2013; Ercan et al., 2013; León et al., 2007). These authors concluded that the presence of oral bacteria in the placenta may be normal, although the levels of certain oral pathogens in the

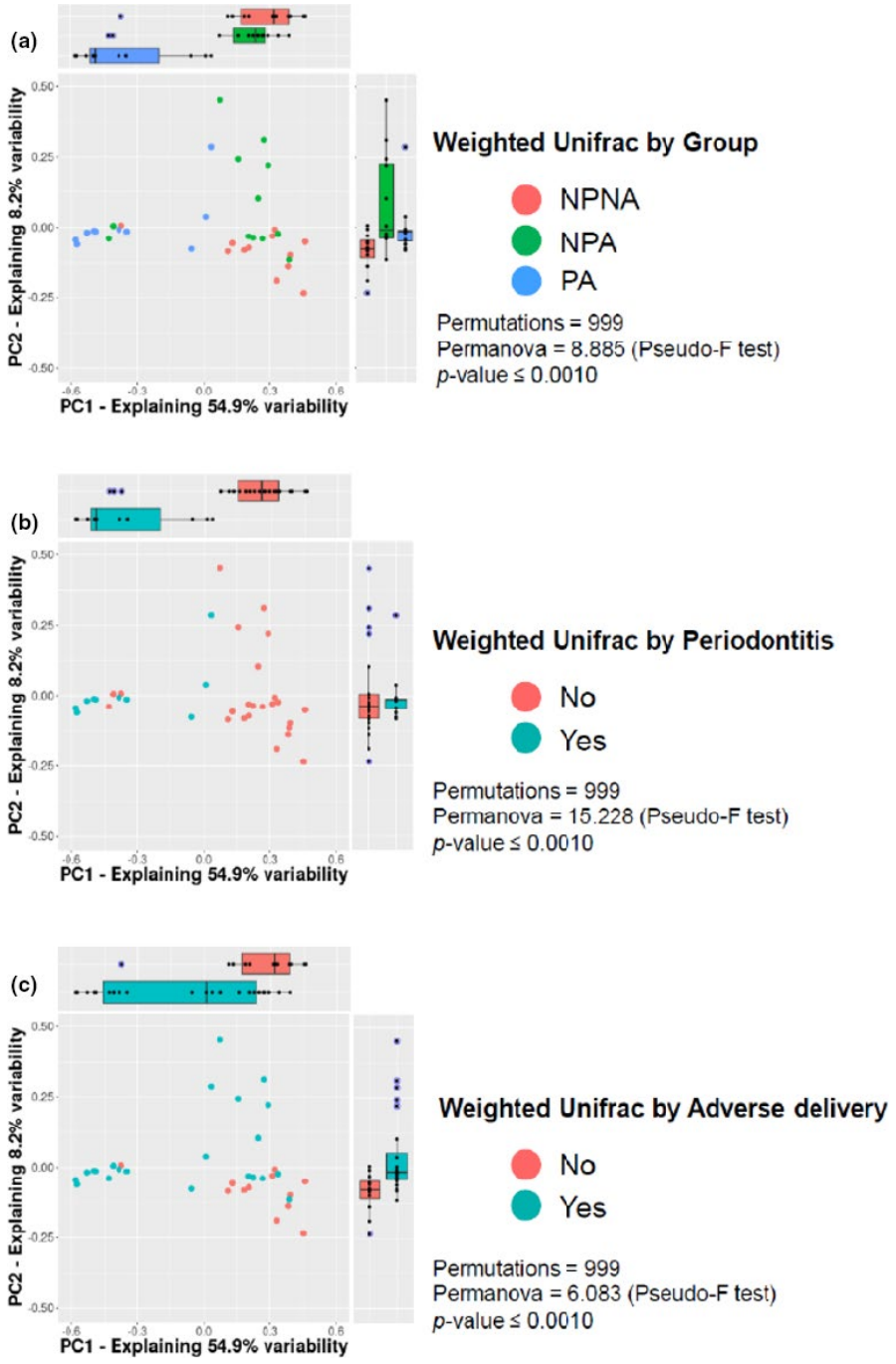

FIGURE 2 Beta diversity assessment. The principal coordinate analysis (PCoA) plots derived from weighted UniFrac distance analysis is present in the scatter plots accompanied with marginal boxplots distribution of data points. The percentage of variation explained for each PC (principal coordinate) is depicted in the x and y axis. Different comparisons were made taking into account the categorical variables of coupled grouping (A), periodontitis status (B), and adverse delivery (C). Color legends are shown in every case. Statistical estimates and p-values are shown accordingly

placenta may be highly dependent on the mother's periodontal disease. However, with the use of high-throughput DNA sequencing methods, it was shown that the placenta and amniotic fluid of healthy pregnant women harbor a unique low-abundance microbial community, and that this microbiota was highly similar to the oral microbiome in pregnancy (Aagaard et al., 2014; Collado et al., 2016; Gomez-Arango et al., 2017). In particular, the study by Gomez-Arango et al. provides evidence that both the maternal oral and gut microbiome may contribute to the seeding of the placental microbiome (Gomez-Arango et al., 2017).

In line with previous reports in the literature, our study suggested that there was a low microbial load in all three groups of placentas evaluated (PA, NPA, and NPNA). Furthermore, this microbiota could be clearly differentiated from the contamination controls before and after applying the corrections for the elimination of background noise (Figure 1). In parallel, we observed that the microbiota of placentas from women with full-term deliveries (NPNA) differed from that present in mothers with adverse pregnancy outcomes (PA and NPA), in agreement with the studies by Aagaard et al., 2014, Prince et al., 2016 and Seferovic et al., 2019. Interestingly, when analyzing the three populations in terms of both variables (periodontitis and adverse pregnancy outcomes), periodontitis appeared to influence the microbial composition of the placentas (Figure 2B and 2C). Furthermore, the microbiotas of the

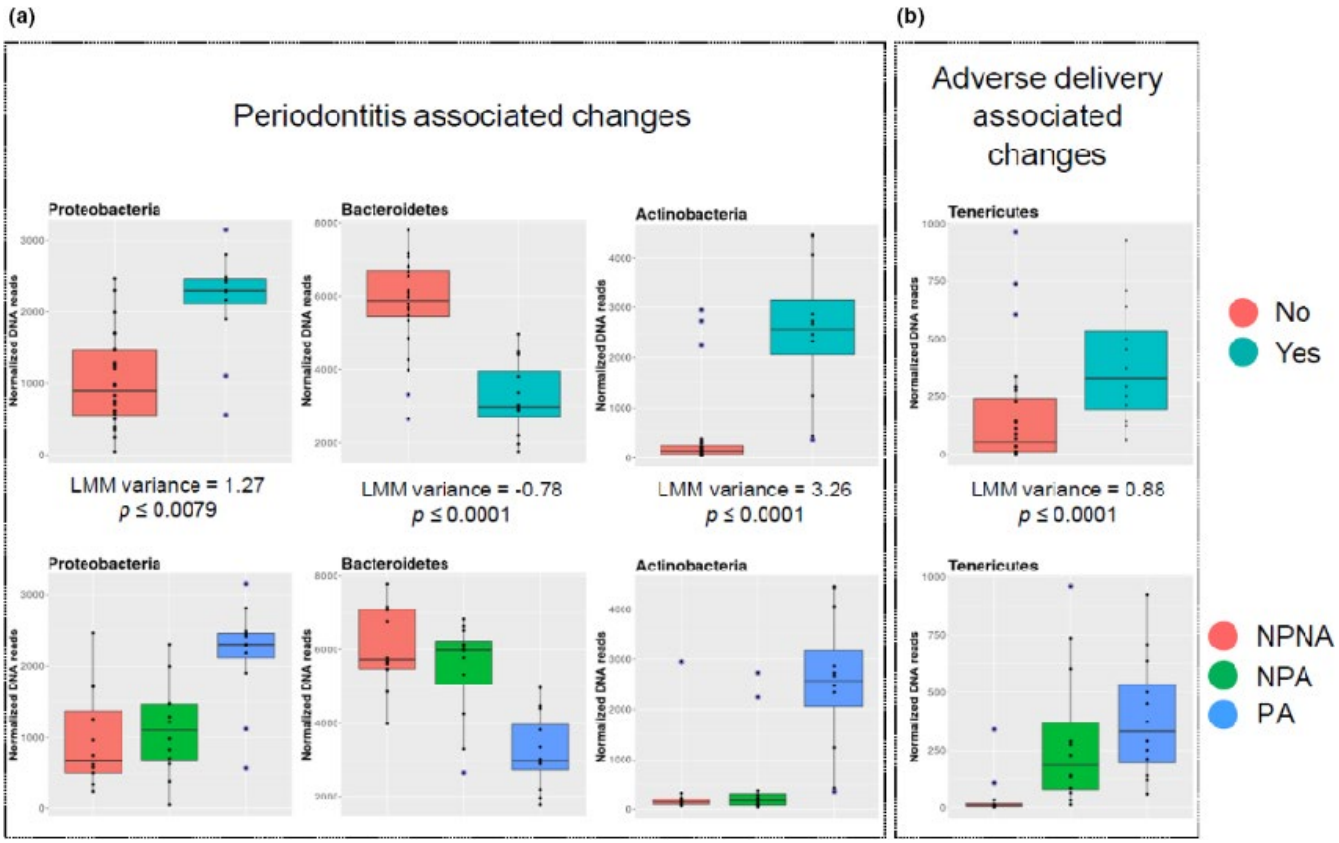

FIGURE 3 Taxonomy assessment at phylum level. Taxonomy features with significant changes across groups are plotted in boxplot fashion. A—The set of taxonomy groups associated with periodontitis status (no/yes). B—Taxonomy category associated with adverse delivery (no/yes). Color legends are shown in every group arrangement. Statistical estimates and p-values are shown accordingly

groups without periodontitis (NPNA and NPA) were more similar to one another, confirming the discriminative power of this disease in the placental microbiota of mothers suffering from adverse pregnancy outcomes (Figure 2A).

In the three groups of placentas studied, bacteria belonging to at least five phyla were detected: *Proteobacteria*, *Bacteroidetes*, *Actinobacteria*, *Tenericutes*, and *Firmicutes*. Particularly, *Proteobacteria*, and *Actinobacteria* were strongly associated with periodontitis. Some of these findings differ partially from those reported in a systematic review of the periodontitis microbiome in which phyla such as *Bacteroidetes*, *Spiroquetes*, and *Firmicutes* were primarily associated with periodontitis (Pérez-Chaparro et al., 2014). Furthermore, in our study, the phylum *Tenericutes* was found to be more closely associated with adverse pregnancy outcomes (Figure 3B). *Mycoplasma hominis*, a representative of this phylum, has been widely associated with placental tissue infections and may use an ascending route to colonize these tissues (Jajoo et al., 2020). In our study, we found the genus *Mycoplasma* to be strongly associated with adverse pregnancy outcomes and also with periodontitis. In other studies, however, *Porphyromonas gingivalis* was less prevalent in placentas with pregnancy complications than in the groups without adverse pregnancy outcomes (Montenegro et al., 2019).

Authors of similar studies have reported the presence of a greater number of phyla. However, caution should be exercised when considering direct comparison of the results, since the use of different regions of the 16S rRNA gene may have yielded different sequencing patterns.

Six families of the phylum *Actinobacteria*, seven of the phylum *Firmicutes*, and 11 of the phylum *Proteobacteria* were found to be positively associated with periodontitis. Surprisingly, the *Porphyromonadaceae* family was not among them (Table 1). *P. gingivalis* belongs to this taxonomic category and is an important periodontopathogen present in most cases of periodontitis (Rylev & Kilian, 2008). Furthermore, several authors have reported its presence in the amniotic fluid and the placentas of mothers who have periodontitis and adverse pregnancy outcomes (León et al., 2007; Parthiban et al., 2018). It is thought that the high capacity of this bacterium to induce inflammation could generate an adverse effect on the placenta through a range of pathways. One of these pathways may involve the release of IL-8 and IFN-gamma in the cells that make up the extravillous trophoblasts, inducing inflammation, apoptosis, and a malfunction of the trophoblast, which would activate other pathways that may converge and ultimately cause PTB (Ren et al., 2016).

TABLE 2 Bacterial families associated with periodontitis and adverse delivery variables

Family	Phylum	Periodontitis Variance[a]	Periodontitis *p*	Adverse_delivery Variance[a]	Adverse_delivery *p*
Rubrobacteraceae	Actinobacteria	5.182	0.0001	1.037	0.1965
Propionibacteriaceae	Actinobacteria	4.993	0.0001	2.514	0.025
Microbacteriaceae	Actinobacteria	4.485	0.0001	1.449	0.1258
Staphylococcaceae	Firmicutes	4.382	0.0001	2.395	0.0132
Burkholderiaceae	Proteobacteria	4.356	0.0001	1.094	0.1624
Micrococcaceae	Actinobacteria	4.247	0.0001	1.017	0.2331
Neisseriaceae	Proteobacteria	4.032	0.0012	1.768	0.1283
Moraxellaceae	Proteobacteria	3.935	0.0001	1.541	0.0959
Corynebacteriaceae	Actinobacteria	3.926	0.0001	2.106	0.0241
Pasteurellaceae	Proteobacteria	3.784	0.0001	0.885	0.3083
Chitinophagaceae	Bacterioidetes	3.758	0.0001	0.364	0.6724
Bifidobacteriaceae	Actinobacteria	3.669	0.0005	1.148	0.2378
Carnobacteriaceae	Firmcutes	3.622	0.0004	0.733	0.432
Sphingomonadaceae	Proteobacteria	3.532	0.0000	0.557	0.4543
Pseudomonadaceae	Proteobacteria	3.530	0.0000	1.884	0.0178
Comamonadaceae	Proteobacteria	3.513	0.0001	1.564	0.0593
Caulobacteraceae	Proteobacteria	3.345	0.0001	0.685	0.4043
Rhodobacteraceae	Proteobacteria	3.197	0.0001	0.467	0.5196
Mycoplasmataceae	Tenericutes	3.183	0.0001	2.861	0.0023
Enterococcaceae	Firmcutes	3.162	0.0001	1.381	0.1257
Colstridiales_Incertae_Sedis_XL	Firmcutes	3.140	0.0004	0.161	0.8422
Xanthomonadaceae	Proteobacteria	3.131	0.0002	1.118	0.1391
Streptococcaceae	Firmcutes	3.043	0.0007	2.395	0.0061
Aerococcaceae	Firmcutes	2.976	0.0013	0.908	0.2922
Alcaligenaceae	Proteobacteria	2.670	0.0064	0.707	0.4465
Actinomycetaceae	Actinobacteria	2.641	0.0018	0.925	0.2435
Bacillales_Incertae_Sedis_XL	Firmcutes	2.463	0.0095	0.754	0.4051
Parachlamydiaceae	Chlamydia	1.812	0.0078	0.151	0.8153
Ruminococcaceae	Firmcutes	−0.384	0.0075	0.065	0.6305
Porphyromonadaceae	Bacterioides	−1.189	0.0004	−0.097	0.7496
Rhodospirillaceae	Proteobacteria	−2.319	0.0086	2.129	0.015
Anaeroplasmataceae	Tenericute	−2.615	0.0095	2.994	0.0034

[a]Variance referred to the "Yes" events for periodontitis and adverse delivery variables. Positive values indicate significantly higher occurrence of such categories in samples associated with "Yes" events. Negative values indicate significantly higher occurrence in samples associated with "No" events.

In the placenta samples, several different bacterial genera were identified, of which the following commonly reside in the oral cavity: *Veillonella*, *Corynebacterium*, *Streptococcus*, *Actinomyces*, *Propionibacterium*, *Haemophilus*, *Enterococcus*, *Neisseria*, *Gemella*, among others. Of these, only *Streptococcus* was found to be associated with periodontitis and adverse pregnancy outcomes. Other authors have also described the presence in placenta of the genera *Rothia*, *Haemophilus*, *Veillonella*, and *Streptococcus*, although the last two usually occur in healthy women (Collado et al., 2016; Gomez-Arango et al., 2017). On the other hand, in a mouse model, tail vein injection of bacteria from the genera *Veillonella* and *Streptococcus* caused the translocation of these species to the placenta (Fardini et al., 2010).

In the present study, the genera associated with adverse pregnancy outcomes and periodontitis were *Holdemanella*, *Mycoplasma*, and *Streptococcus*. The first of these is normally associated with intestinal dysbiosis, and the last two are possibly of oral origin (Gomez-Arango et al., 2017; Huang et al., 2020).

This study has some limitations that should be mentioned. First, the placenta samples being embedded in paraffin may have limited the amount of DNA extracted. Clearly, this may have prevented the detection of species that were present in very low numbers.

TABLE 3 Bacterial genus associated with periodontitis and adverse delivery variables

Genus	Periodontitis Variance	Periodontitis *p*	Adverse_delivery Variance	Adverse_delivery *p*
Rubrobacter	5.182	0.0001	1.037	0.1965
Propionibacerium	5.007	0.0001	2.512	0.0255
Haemophilus	4.526	0.0001	1.662	0.0814
Ralstonia	4.508	0.0001	0.865	0.2416
Staphylococcus	4.455	0.0001	2.332	0.0138
Cotynebacterium	3.899	0.0001	2.066	0.0232
Blautia	3.743	0.0001	1.325	0.097
Psychorobacter	3.702	0.0001	1.374	0.0718
Rothia	3.664	0.0001	1.123	0.1784
Bifidobacterium	3.599	0.0006	1.214	0.2123
Holdemanella	3.427	0.0001	2.317	0.0002
Pseudomonas	3.406	0.0001	1.935	0.0115
Mycoplasma	3.342	0.0006	2.808	0.0032
Veillonella	3.313	0.0004	0.583	0.4887
Sphingomonas	3.276	0.0001	0.072	0.9223
Enterococcus	3.154	0.0009	1.321	0.1363
Olsenella	3.136	0.0009	-0.292	0.7358
Megamonas	3.116	0.0014	1.743	0.0599
Faecalibacterium	3.094	0.0006	1.228	0.1423
Micrococcus	3.09	0	-0.022	0.9714
Acinetobacter	3.04	0.0006	0.29	0.7207
Streptococcus	3.023	0.0008	2.394	0.0064
Neisseria	3	0.0014	0.857	0.3254
Kandleria	2.995	0.0008	0.224	0.7836
Actinomyces	2.951	0.0008	0.74	0.3598
Collinsella	2.914	0.0021	2.18	0.0175
Phenylobacterium	2.875	0.0018	0.024	0.9779
Gemminger	2.756	0.0037	0.51	0.5669
Pelomonas	2.74	0.001	0.279	0.7168
Moraxella	2.707	0.0046	0.907	0.3165
Paracoccus	2.621	0.0006	0.693	0.3198
Lysinibacillus	2.606	0.0065	0.791	0.384
Stenotrophomonas	2.512	0.0015	0.741	0.3148
Gemella	2.463	0.0095	0.754	0.4051
Ruminococcus2	2.448	0.001	0.46	0.501
Granulicatella	2.4	0.0035	1.132	0.1471
Pasteurella	2.377	0.0046	0.177	0.8224
Anaerococcus	2.344	0.0018	-0.159	0.8196
Microbacterium	2.242	0.0002	0.193	0.7186
Odoribacter	1.857	0.0029	0.215	0.7118
Burkholderia	1.536	0.004	0.686	0.177
Achromobacter	1.48	0.0057	0.000	1
Rhodococcus	1.283	0.0048	−0.083	0.8456
Parachlamydia	0.874	0.0026	0	1
Anaerostipes	0.362	0.6679	3.632	0.0001

(Continues)

TABLE 3 (Continued)

Genus	Periodontitis Variance	Periodontitis *p*	Adverse_delivery Variance	Adverse_delivery *p*
Slackia	0.093	0.8488	−1.868	0.0005
Paraeggerthella	0	1	−0.753	0.0086
Oxalobacter	−1.017	0.1645	−2.023	0.0079
Pseudobutyrivibrio	−1.958	0.0022	0.232	0.697
Clostridium_IV	−2.662	0.0011	1.098	0.1498
Sutterella	−2.924	0.0046	1.835	0.653
Paraprevotella	−3.31	0.0059	1.49	0.1943
Sphaerochata	−3.876	0.0005	0.752	0.4573

[a]Variance referred to the "Yes" events for periodontitis and adverse delivery variables. Positive values indicate significantly higher occurrence of such categories in samples associated with "Yes" events. Negative values indicate significantly higher occurrence in samples associated with "No" events.

Secondly, the reduced number of samples analyzed makes us wary of drawing conclusions, particularly with regard to the classification of the microbiota to the genus level. Thirdly, the transport of fresh tissue samples was ruled out, for logistical reasons. In spite of the strict specimen collection protocol, the long-distance transportation of all samples to the microbiology laboratory might be considered a weak point in the relocation process, but this is something that was beyond our control. These limitations notwithstanding, the study presents two important findings that are worth highlighting. First, the placenta samples from the three groups of pregnant women hosted diverse microbiota, and second, the structure of the microbial community found in the placenta may be strongly conditioned by periodontitis.

In a similar context, Nadal et al. (2019) evaluated the presence and grade of chorioamnionitis in 50 placentas from the Kilimanjaro Christian Medical Centre in Tanzania. The authors concluded that the group of pregnant women with periodontitis presented a higher risk of developing chorioamnionitis, lower birth weight, and lower feto-placental ratio than those without (Nadal et al., 2019). We stress that chorioamnionitis with fetal response is associated with worse overall and neurodevelopmental neonatal prognosis than chorioamnionitis with only maternal response (Kim et al., 2001).

In the last 25 years, several clinical studies have sought to confirm the relationship between maternal periodontitis and PTB or LBW. However, there is still controversy regarding the real risk that a diagnosis of periodontitis entails for a pregnant mother. Some authors attribute this controversy to the use of different clinical criteria to define or characterize periodontitis (Ide & Papapanou, 2013; Moncunill-Mira et al., 2021); it is likely that the new classification of this disease will help to elucidate this situation.

In generalized periodontitis, the sum of the active lesions, with ulcerated epithelium of the periodontal pockets, has been estimated to be equivalent to a wound with a surface area approximately the size of the palm of a hand. This would allow direct contact between the infectious-inflammatory contents of each periodontal pocket and the gingival connective tissue vessels and may favor the systemic spread of bacteria, lipopolysaccharides, and pro-inflammatory cytokines through the bloodstream. (Moncunill-Mira et al., 2021). Certain authors state that cytokines such as IL1β, TNF-α, and IL6 can reach the uterus and pass through the fetal–placental barrier (Van Dyke & van Winkelhoff, 2013). Similarly, in a study measuring and evaluating acute-phase proteins in serum, saliva and umbilical cord. Al Rawi et al. (2021) suggested that pregnant women exhibiting severe periodontitis, together with upregulation of IL6, are potentially prone to preterm birth.

A recent study by Gomez et al. came to the conclusion that periodontitis might be associated with clinical signs of placental infection and adverse pregnancy outcomes. These authors stated that periodontal infection by *P. gingivalis* can induce atopobiosis (translocation) to the placenta and trigger inflammation, although no direct relationship with the occurrence of adverse pregnancy outcomes could be shown (Gomez et al., 2020).

Our study corroborates the presence of a low microbial load in the placenta of mothers with different periodontal conditions and adverse pregnancy outcomes. The presence or absence of periodontitis produced a clear differentiation in the microbiota of these placentas, and several oral bacterial genera were found to be associated with these adverse pregnancy outcomes. The ability of bacteria in the oral cavity to reach the placental tissues underpins the need to monitor women's oral health before and during pregnancy. In the future studies the clinical assessment of maternal periodontitis and some adverse obstetric and fetal outcomes could be complemented by analyzing the most characteristic inflammatory mediators related to periodontal disease in the maternal plasma serum.

We conclude that periodontal pathogens may involve the feto-placental unit, and the structure of the microbial community found in the placenta may be strongly conditioned by periodontitis. Maternal periodontal status was associated with a clear differentiation in the microbiota of the placentas, and several oral bacteria present in this niche were associated with adverse pregnancy outcomes.

5 | ETHICAL CONSIDERATION

Ethical clearance certificate No. 822/2015 was obtained from the Kilimanjaro Christian Medical University College Research and

Ethics Committee. Study participants signed an informed consent form before data collection, and all the information was treated confidentially. Participants who did not provide consent received the same care as those who did.

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CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

AUTHOR CONTRIBUTIONS

Jaume Miranda-Rius: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Writing-review & editing. **LLuís Brunet-Llobet:** Conceptualization, Data curation, Formal analysis, Investigation, Project administration, Supervision, Writing-review & editing. **Vanessa Blanc:** Conceptualization, Formal analysis, Investigation, Methodology, Resources, Software, Supervision, Validation, Visualization, Writing-original draft. **Gerard Álvarez:** Data curation, Investigation, Methodology, Software. **Jordi Moncunill-Mira:** Data curation, Formal analysis, Investigation. **Elias Mashala:** Data curation, Methodology. **Yona Kasabele:** Data curation, Resources. **Gileard Masenga:** Data curation, Resources. **Alfons Nadal:** Data curation, Formal analysis, Investigation, Methodology, Resources, Writing-original draft. **Ruben Leon:** Formal analysis, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Writing-original draft.

PEER REVIEW

The peer review history for this article is available at https://publons.com/publon/10.1111/odi.13962.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the last author and the corresponding author upon reasonable request.

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