Orofacial Clefts Alter Early Life Oral Microbiome Maturation Towards Dysbiosis

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Article

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Abstract

Orofacial clefts (OFC) present different phenotypes and severities with a postnatal challenge for oral microbiota development. In order to investigate the impact of OFC on oral microbiota, smear samples from 15 neonates with OFC and 17 neonates without OFC were collected from two oral niches (tongue, cheek) at two time points, i.e. at first consultation after birth (T0: Ø3d OFC group; Ø2d control group) and 4–5 weeks later (T1: Ø32d OFC group; Ø31d control group). Subsequently, the samples were processed and analyzed using next-generation sequencing. We detected a significant increase in alpha diversity and distinct phenotypes, e.g., *anaerobic* and *gram-negative species* from T0 to T1 in both groups. Further, we found that at T1 OFC neonates presented a significantly lower alpha diversity with lowest values for high cleft severity and significantly higher levels of *Enterobacteriaceae (Citrobacter, Enterobacter, Escherichia-Shigella, Klebsiella), Enterococcus, Bifidobacterium, Corynebacterium, Lactocaseibacillus, Staphylococcus, Acinetobacter and Lawsonella* compared to controls. Notably, neonates with unilateral and bilateral cleft lip (UCLP/BCLP) and palate presented similarities in beta diversity and a mixture with skin microbiota. However, significant differences were seen in neonates with cleft palate only compared to UCLP/BCLP with higher levels of *anaerobic species*. Our findings revealed an influence of OFC as well as cleft phenotype and severity on postnatal oral microbiota maturation.

Introduction

Cleft lip and palate (CLP) are the second most common human malformation and show a varying prevalence of approximately 1:700 for cleft lip palate and 1:1200 for cleft palate only depending on ethnic group, cleft type/side and gender [1]. They present different phenotypes, e.g., bilateral cleft lip and cleft palate (BCLP), unilateral cleft lip and cleft palate (UCLP), Cleft palate only (CPo), Cleft lip only (CLo) [2]. Orofacial clefts are the result of maxillofacial processes between the 7th and 12th embryonic week [3]. Due to the variety of cleft types and severities, several classification schemes have been evolved [2,4], e.g., the LAHSHAL classification scheme by Kriens *et al*. [5], which is the second-most common and recommended classification scheme due to its adequateness and extensiveness [4]. Depending on cleft severity, neonates with OFC require surgical lip and/or surgical palate closure [6,7] as well as presurgical infant orthopedics (PSIO) with palate plates [8], which aim to control growth, ensure feeding and normalize function [7,9]. Despite PSIO treatment feeding difficulties [10,11] can still occur in neonates with OFC requiring nutrition interventions like Haberman feeder [12,13].

Physiologically, the oral and nasal cavity are solely connected over the nasopharyngeal zone and separated by the hard and soft palate as well as the alveolus and the upper lip, however, depending on the severity of the cleft, both cavities can be joined over smaller to larger areas [2]. This could lead to an unphysiological communication of the oral and nasal microbiome. The oral microbiota displays the second-largest human microbiota after the gut and is composed of up to 775 bacterial taxa in adults including 57% named species, 30% uncultivated species and 13% cultivated unnamed taxa with a provisional name based on the 16S rRNA sequence [14]. Considering initial maturation, some authors showed that the intrauterine environment is sterile indicating that the maturation of the human microbiota begins after birth [15,16], while others demonstrated similarities between the neonatal oral and mothers placental microbiota [17,18] indicating that development of oral microbiota begins during pregnancy. The oral cavity contains anatomically different niches, which partly develop in the embryonic phase and fetal period, e.g., the tongue (T), the soft and hard palate (HP), the cheeks (C) and sublingual area (U), and partly after birth and during childhood, e.g., teeth, the gingival sulcus with the gingival crevicular fluid (GCF) and dental plaque (P). Interestingly, in young periodontal healthy adults a clustering into three microbial metaniches was detected, that can be explained by anatomical proximity, function, salivary glands and saliva flow rate [19]. Regarding neonates with orofacial clefts, it has been shown that 17 months old infants with non-syndromic OFC presented a lower oral microbial diversity and an altered microbial composition compared to controls before surgical palate closure [20]. Regarding different cleft types, distinct oral bacterial species were investigated using culture analyses

in neonates with CLP and CPo at two time points (1–2 and 8–18 weeks after birth) and significant differences were seen between the cleft types and between the time points [21]. Contemplating possible changes before and after surgical cleft closure, a decrease of certain bacterial genera was seen in CLP neonates after palate closure [22]. Moreover, in 10-year-old adolescents with CLP, it has been shown that the abundance of specific bacterial species prior to alveolar bone grafting correlated with post-surgical inflammation at operative sites [23]. Further, there is some evidence that dysbiosis of the gut microbiome in neonates may be associated to vulnerability for inflammatory diseases in childhood [24]. Hence, disturbances of oral microbiome development may be a risk factor for early life dysbiosis, inflammation and in consequence might be linked to adverse surgery outcomes and dysbiotic inflammatory diseases later in life.

Yet, studies investigating an early impact of oral microbiota maturation in neonates with OFC compared to an age-matched control group and regarding oral niches using next generation sequencing (NGS) are missing. The knowledge about possible early developmental alterations in oral microbiota in neonates with different CLP types might be crucial for future studies investigating risk factors for wound healing disorders within the first year of life and oral inflammatory diseases even later on. Therefore, we analysed the impact of OFC on oral microbiota in 15 neonates with OFC and 17 neonates without OFC using next-generation sequencing.

Results

Oral microbial composition showed a distinct clustering in neonates with OFC and control neonates after the first weeks of life

Microbiota analyses of 126 oral samples from two groups (CLP vs. control), two time points, i.e. at first consultation after birth (T0: Ø3d CLP; Ø2d control) and 4–5 weeks later (T1: Ø32d OFC; Ø31d control) and two different oral niches (T, C) revealed four microbial phyla, nine classes, 20 orders, 30 families, and 39 genera derived from 117 operational taxonomic units (OTU). Comparing the distribution of microbial genera for both niches (niche T vs. C) and different combinations of site, group and time point, no distinct clusters were detectable for T or C. Both niches were previously found in young adults with periodontal health to be part of different metaniches with distinct microbial composition (10). However, a dendrogram based on generalized UniFrac distances showed clustering into three branches (Fig. 1). The lower branch represented a cluster mainly formed by samples of the CLP and control group at time point T0 collected from both niches T and C (Fig. 1). Regarding the lowest branch, a trend to a separation into two clusters formed by the CLP and control group can be seen, however, those clusters were not clearly distinguishable (Fig. 1). The middle branch represents a distinct cluster for the CLP group at time point T1 without differences in oral niches (Fig. 1). Control samples from time point T1 including both niches clustered in the upper branch (Fig. 1). Here, also two patients of the CLP group clustered: LKG-002 and LKG-018, both presenting only mild phenotypic manifestations (Table 1). Since LKG-018 was the only neonate affected by CLo and, therefore, the only patient without clefting of intraoral structures (soft/hard palate and alveolous) and a potential mixture of nasal and oral microbiota, it was excluded in further investigations referring to the complete CLP group. LKG-002 was still included in further investigations due to the affection of intraoral structures (soft/hard palate).

Table 1
Study population

Characteristics of the control group									
#	Age at T0 (d)	Age at T1 (d)	Gender	Weight T0 (g)	Height T0 (cm)	Type of birth	PROM	Antibiotics	Nutrition[1]
LKGc-001	2	36	f	3350	52	c	0	ABM	0
LKGc-002	2	34	m	3800	53	c	0	ABM	0
LKGc-003	2	27	m	4120	55	c	0	ABM	0
LKGc-004	2	23	f	3150	50	c	1	ABM	0
LKGc-005	2	32	m	3340	50	v	0	noABM	0
LKGc-008	3	29	f	3180	51	v	1	noABM	0
LKGc-009	2	35	m	4030	54	v	0	noABM	0
LKGc-010	2	38	m	3640	53	v	0	noABM	0
LKGc-011	2	31	m	3050	50	v	1	noABM	0
LKGc-012	2	20	f	2930	50	v	1	noABM	0
LKGc-013	3	32	m	3670	54	v	1	noABM	0
LKGc-015	3	34	f	3200	50	v	1	noABM	0
LKGc-016	2	42	m	3940	53	c	1	ABM	0
LKGc-018	2	24	f	3570	54	v	0	noABM	0
LKGc-020	3	36	f	4200	56	c	0	ABM	0
LKGc-021	3	22	m	3480	54	c	0	ABM	0
LKGc-022	3	24	m	2950	50	v	0	noABM	0
Characteristics of the study group (CLP)									
#	Age at T0 (d)	Age at T1 (d)	Gender	Weight T0 (g)	Height T0 (cm)	Type of birth	PROM	Anti-biotics	Nutrition [1]

Characteristics of the control group									
LKG-001	7	24	m	2590	48	v	0	noABM	2
LKG-002	n.d.	19	m	3130	44	c	0	ABM	2,*4*
LKG-003	3	38	m	3040	51	c	0	ABM	2
LKG-004	5	n.d.	f	2980	51	v	0	noABM	0
LKG-005	2	37	f	2940	51	v	0	noABM	1
LKG-007	2	29	m	3220	51	v	0	noABM	1
LKG-009	3	29	m	3240	49	v	0	ABM	3
LKG-010	3	38	m	3320	51	c	0	ABM	3,*4*
LKG-011	3	34	m	3350	53	v	0	noABM	1
LKG-012	3	25	m	3900	51	v	0	noABM	2,*4*
LKG-014	2	31	m	3120	47	c	0	ABM	2
LKG-015	1	22	f	2800	51	v	0	noABM	2
LKG-016	11	39	f	3120	51	v	0	noABM	1,*4*
LKG-017	14	40	m	2860	50	v	0	noABM	3
LKG-018	8	34	f	3890	51	v	0	noABM	0
Special characteristics of the study group: classification, severity, type of treatment									
#	Etio-	BCLP	UCLP*	CPo	CLo	LAHSHAL	LAHSHAL	Severity	pAM
	logy					Code [2]	Severity[3]	Score[4]	
LKG-001	ps	0	1	0	0	- - - SHAL	0002222	8	1
LKG-002	s	0	0	1	0	- - hSh - -	0012100	4	0
LKG-003	ps	0	1	0	0	- - - SHAL	0002222	8	1
LKG-004	s	0	0	1	0	- - hSh - -	0012100	4	0

Characteristics of the control group									
LKG-005	s	0	0	1	0	- - HSH - -	0022200	6	1
LKG-007	ps	0	1	0	0	- - - SHAl	0002221	7	1
LKG-009	ps	1	0	0	0	lAHS - - l	1222001	8	1
LKG-010	ps	1	0	0	0	LAHSHAL	2222222	14	1
LKG-011	ps	0	1	0	0	LAHS - - -	2222000	8	1
LKG-012	ps	1	0	0	0	LAHSHAL	2222222	14	1
LKG-014	ps	0	1	0	0	lAHS - - -	1222000	7	1
LKG-015	ps	1	0	0	0	laHSHAL	1122222	12	1
LKG-016	s	0	0	1	0	- - HSH - -	0022200	6	1
LKG-017	s	0	0	1	0	- - HSH - -	0022200	6	1
LKG-018	p	0	0	0	1	la - - - - -	1100000	2	0

0 = no, 1 = yes, s = cleft of the secondary palate, p = cleft of the primary palate, ps = cleft of the primary and secondary palate, d = days, g = grams, cm = centimeter, f = female, m = male, n.d. = not done, c = Caesarian section, v = vaginal, PROM = premature rupture of membranes, noABM = no antibiotic intake of mother intrapartum, ABM = antibiotic intake mother intrapartum, pAM = passive alveolar molding, T0 = time point T0 after birth, T1 = Time point T1 4–5 weeks after birth, BCLP = bilateral cleft lip palate, UCLP = unilateral cleft lip palate, CPo = Cleft Palate only, CLo = Cleft Lip only.

[1] 0 = breastfeeding, 1 = bottle feeding breast milk, 2 = bottle feeding partly breast milk, partly artificial formula, 3 = bottle feeding artificial formula, 4 = postnatal tube feeding for < 1 week (= T0);

[2] LAHSHAL Code: minus sign (-) = not affected, small letter = incompletely affected, capital letter = completely affected

[3] LAHSHAL Severity: 0 = not affected, 1 = incompletely affected, 2 = completely affected

[4] Severity Score: sum of the LAHSHAL Severity

After birth (T0), oral microbial composition in neonates from both groups was dominated by the following genera (CLP; control): *Streptococcus* (68.03%; 59.18%), *Staphylococcus* (15.84%; 8.70%), *Gemella* (4.88%; 12.17%) and *Rothia* (8.36%; 3.67%). *Haemophilus* and *Neisseria* solely presented higher levels in the control group (12.02%; 3.88%) and all other genera, detected in isolated samples, ranged below 1% (Fig. 1). The mean highest abundance of *Streptococcus* was most impressive in one CLP neonate (LKG_014_T0) presenting a mono colonization with this genus (Fig. 1).

Within the first weeks of life (T1), high abundance of *Streptococcus* (65.01%; 66.58%), *Staphylococcus* (4.72%; 4.34%), *Gemella* (1.97%; 4.84%) and *Rothia* (4.36%; 2.68%) the subsequent genera were still detected in both groups (CLP; control) (Fig. 1). However, a higher variety of genera was found in both groups. The control group presented elevated

levels of *Haemophilus* (6.21%), *Veillonnella* (7.18%), *Lactobacillus* (1.06%), Prevotella 7 (1.93%), *Atopobium* (1.61%) and *Actinomyces* (1.54%) (Fig. 1). The CLP group showed increased levels of *Bifidobacterium* (7.94%), *Neisseria* (0.03%; 3.88%), *Escherichia-Shigella* (1.99%), *Lactobacillus* (1.60%), *Limosilactobacillus* (2.34%), *Enterococcus* (2.45%), *Bergeyella* (1.20%) and *Corynebacterium* (1.15%) (Fig. 1).

Taken together, the oral microbial composition of CLP and control neonates after birth (T0) was similar and dominated by *Streptococcus* spp. without distinct clustering according to group or site. Concerning both groups after 4–5 weeks of life (T1), a greater microbial variety and distinct formations of oral microbiota were seen for neonates with orofacial clefts and neonates without CLP based on abundance of microbial genera. Notably, metaniche-characteristics [19] were not seen in both groups after birth and within the first weeks of life.

Alpha diversity was significantly higher in control neonates compared to CLP neonates in the niche tongue at both time points

Together with patient LKG-002, we found patient LKG-018 to cluster with the control group at T1 (Fig. 1). Because of its relatively mild CLP manifestation (LAHSHAL: la - - - - -) and no affection of intraoral structures we excluded patient LKG-018 in the following group comparisons to facilitate the identification of CLP-specific differences. Considering both sampling niches for calculating alpha diversity, we observed a significantly lower alpha diversity of tongue samples in CLP neonates compared to control neonates at both time points (Fig. 2a). Except for cheek samples of the control group, we further observed a significant increase in alpha diversity from T0 to T1 in all groups (Fig. 2b). To evaluate the impact of CLP severity on a more granular, individual base, we transformed the LAHSHAL code in a severity score using numbers (see Methods section). Interestingly, significant differences were seen at T0 with a higher alpha diversity in control neonates compared to CLP neonates with high severity scores (Fig. 2c). CLP neonates with low severity scores also presented a higher alpha diversity compared to CLP neonates with high severity scores at T0 (Fig. 2c). Notably, significant differences between the groups levelled out at T1 (Fig. 2c). Previous data suggested an impact of birth type on the oral microbiome [25]. Again, we combined both sampled niches to increase the number of samples per group. The control group presented a significantly higher alpha diversity in neonates born via C-section at T0, while no differences were seen for CLP neonates and at T1 for both groups (Fig. 2d) indicating that differences between the control and CLP group are not due the different birth modes.

Summarizing, we demonstrated a significant (physiological) increase of alpha diversity from T0 to T1 in both groups. The presence of orofacial clefts resulted in lower alpha diversity in the niche tongue at both time points and in both niches combined at T0. High cleft severity inhibited the (physiological) increase in alpha diversity the most.

Ordination analyses using multidimensional scaling reveals significant differences in the development of beta diversity within the first days of life in both groups

In order to investigate beta diversity, ordination using multidimensional scaling (MDS) based on generalized UniFrac distances of log-transformed genus counts were performed visualizing 44% (Fig. 3a and c) and 50.7% (Fig. 3b) of whole data variability. Applying permutational multivariate analysis of variance (PERMANOVA) via the adonis2 test (vegan package for R), no significant differences were seen with a combination of sample niche (T vs. C), patient group and time point (Fig. 3a) variables. However, using the same test significant differences ($p = 0.001$) were obtained with the combination of group and time point variable only. Consequently, ignoring sampling niche for grouping, we observed only minor differences in beta diversity for both groups at T0 (pairwise adonis2 p adj. = 0.018) with both clusters largely overlaying each other (Fig. 3c). However, we could show more significant differences between the CLP and the control group at T1 (pairwise adonis2 p adj. = 0.006) with noticeable separation of both clusters (Fig. 3c). To demonstrate this more clearly, we performed constrained correspondence analysis (CCA) of the same data with constriction to group and time point (Fig. 3d). Representing 16.8% of whole data variability, a clear separation of the CLP group from the control

was evident at T1 (Fig. 3d). In contrast, the clusters of CLP and control group at T0 were in close proximity to each other and exhibited intersecting areas with the cluster of control group samples at T1 (Fig. 3d). A permutation test (anova.cca, vegan package for R) proofed significant ($p = 0.001$) for both depicted constrained axes. For investigation of cleft types, the CLo patient (LKG-018) was included. Notably, the clusters of the UCLP and BCLP group presented almost absolute overlapping at T0, while the CPo group was slightly differentiated from all other categorical groups (Fig. 3b). At T1, the CPo group showed cluster separation with only some overlap with the UCLP and BCLP group (Fig. 3b). Interestingly, the UCLP and BCLP group showed crisscrossing areas at T0 and formed a distinct group with almost completely superimposed ellipses at T1 (Fig. 3b). At T0, the CLo patient presented similar beta diversity than all other categorical groups, however, separated clearly from the UCLP/BCLP cluster and slightly from the CPo cluster at T1 (Fig. 3b).

To summarize, we detected significant differences in beta diversity between T0 and T1 in both groups. Interestingly, cluster segregation was seen for the CLP group at T1. Moreover, beta diversity detected in UCLP and BCLP was similar at T1, however, the CPo group presented distinct differences at T1 and the CLo patient separated from all categorical groups at T1. Similar to alpha diversity, no differences depending on sampling niche were observed with regard to beta diversity.

High level phenotype analyses present higher levels of gram-positive and lower levels of gram-negative species in CLP neonates compared to controls

For investigation of different high-level phenotypes, the identified microbial communities were subjected to BugBase [26] analysis, which is based on PICRUSt [27]. Analyses were done with sample grouping according to group (CLP vs. control) and time point (T0 vs. T1) (Fig. 4) and regarding different cleft phenotypes (CTRL vs. CLo vs. CPo vs. UCLP vs. BCLP) and severities (CTRL vs. CLP low severity vs. CLP high severity) (Fig. 5).

Considering longitudinal changes, a significant ($p < 0.001$) increase of *anaerobic species* was seen from T0 to T1 in both groups (Fig. 4a) and regardless of cleft phenotype and severity (Fig. 5a, f). Regarding neonates with orofacial clefts, *gram-negative species* decreased significantly ($p < 0.05$), while in contrast *gram-positive species* increased significantly ($p < 0.05$) from T0 to T1 in CLP neonates (Fig. 4d, e) regardless of cleft phenotype and severity (Fig. 5d, e, i, j). Controls presented similar but not significant proportional adaptations. Further, a significant ($p < 0.001$) increase of *biofilm forming species* was only seen in CLP neonates from T0 to T1, while *stress-tolerant species* solely increased in control neonates ($p < 0.05$) (Fig. 4e, f).

Differences between both groups were seen at T0 with higher levels of *facultative anaerobic* ($p < 0.001$) and *gram-negative species* ($p < 0.01$) in controls and higher levels of *gram-positive species* ($p < 0.05$) in CLP neonates (Fig. 4b, c, d) regardless of cleft phenotype and severity (Fig. 5c, d, e, i, j).

Considering different cleft phenotypes and severities, significantly higher levels of *anaerobic species* were seen in controls compared to CLP neonates with low cleft severity ($p < 0.05$) (CLo/CPo: $p < 0.001$/ $p < 0.05$), while CLo/CPo neonates showed higher levels compared to UCLP/BCLP ($p < 0.01$/ $p < 0.05$) neonates at T0 (Fig. 5b, g). Moreover, significant differences were seen regarding *facultative anaerobic species* with significantly higher levels in controls compared to UCLP ($p < 0.001$) and compared to CLP neonates with high cleft severity at T0 ($p < 0.01$) (Fig. 5c, h). Notably, also CLo/CPo neonates presented higher levels of *facultative anaerobic species* compared to UCLP neonates at T0 ($p < 0.01$/ $p < 0.05$) (Fig. 5c). Interestingly, there was a significant ($p < 0.01$/ $p < 0.05$) increase of *facultative anaerobic species* in UCLP and CLP neonates with high and low cleft severity from T0 to T1 resulting in significantly ($p < 0.001$) higher levels of *facultative anaerobic species* in CLP neonates with low cleft severity (CPo: $p < 0.01$) compared to controls at T1 (Fig. 5c). No differences were seen between different cleft phenotypes regarding *gram-negative* and *gram-positive species*, however, neonates with high cleft severity presented significantly lower levels of *gram-negative* and significantly higher levels of *gram-positive species* at T0 compared to controls ($p < 0.05$) (Fig. 5d, e, l, j).

Differential analyses on genus level presented significantly higher levels of Enterobacteriaceae **(**Citrobacter, Enterobacter, Escherichia-Shigella, Klebsiella**)**, Enterococcus, Bifidobacterium, Corynebacterium, Lactocaseibacillus, Staphylococcus, Acinetobacter and Lawsonella **in neonates with CLP after the first weeks of life**

Consistent with the genus composition seen in the dendrogram (Fig. 1), differential analysis on genus level revealed no significant (> 2-fold, adj. p (q) < 0.05) differences between both groups at T0 except for higher levels of *Neisseria* and *Haemophilus* in the control group and higher levels of *Pseudomonas* in the CLP group (Fig. 6a). After the first 4–5 weeks of life significant differences were detectable between the groups: the CLP group presented significantly higher abundance of *Enterobacteriaceae* (*Citrobacter*, *Enterobacter*, *Escherichia-Shigella, Klebsiella*), *Enterococcus*, *Bifidobacterium*, *Corynebacterium*, *Lactocaseibacillus*, *Staphylococcus*, *Acinetobacter and Lawsonella* compared to controls (Fig. 6b), while controls showed higher levels of *Veillonella*, *Bergeyella*, *Actinomyces*, *Haemophilus*, *Atopobium*, *Prevotella*, *Porphyromonas*, *Gemella, Alloprevotella* and *Scardovia* (Fig. 6b). Considering changes from T0 to T1, a significant increase of *Veillonella, Bergeyella*, *Actinomyces*, *Atopobium* and *Pseudomonas* was seen in controls (Fig. 6c) and a significant elevation of *Bifidobacterium*, *Enterococcus*, *Citrobacter*, *Klebsiella*, *Corynebacterium* and *Lacticaseibacillus* was seen in the CLP group (Fig. 6d).

Differential analyses on OTU level revealed similarities between neonates with UCLP and BCLP and significant differences in neonates with CPo compared to UCLP/BCLP

Differential analyses on OTU level presented differences with regard to CLP type (CPo, UCLP, BCLP) considering both time points (T0, T1). At both time points, almost no significant differences were detectable between BCLP and UCLP neonates except for elevated levels of *Streptococcus oralis* (OTU 317) in the BCLP group at T0 (Fig. 7a) and higher proportions of *Lactobacillus gasseri* (OTU 12) at T1 (Fig. 7b). However, differences were more evident between CPo and UCLP/BCLP neonates.

Neonates with CPo presented significantly higher levels of *Streptococcus* sp. (OTU 1005; OTU 1834; OTU 1335; OUT 91) compared to UCLP and BCLP neonates at T0 (Fig. 7c, e) as well as elevated numbers of *Staphylococcus hominis* (OTU 2), *Staphylococcus aureus* (OTU 6), *Streptococcus oralis* (OTU 317), *Granulicatella adiacens* (OTU 48) and *Veillonella* sp. (OTU 26) compared to UCLP neonates at T0 (Fig. 7e). At T1, neonates with CPo showed significantly increased abundance of *Klebsiella pneumoniae* (OTU_19), *Prevotella histicola* (OTU_21), *Streptococcus lactarius* (OTU_593), *Streptococcus* sp. (OTU_1309, OTU_1005, OTU_378), *Haemophilus parainfluenzae* (OTU_10), *Streptococcus parasanguinis* (OTU_13), *Chryseobacterium* sp. (OTU_43), *Corynebacterium kroppenstedtii* (OTU_28), *Streptococcus oralis* (OTU_317) and *Neisseria sicca* (OTU_75) compared to BCLP (Fig. 7d) and significantly higher numbers of *Campylobacter concisus* (OTU_65), *Staphylococcus aureus* (OTU_6), *Prevotella histicola* (OTU_21), *Pseudomonas panacis* (OTU_33) and *Veillonella atypica* (OTU_25) compared to UCLP (Fig. 7f). Neonates with BCLP showed significantly higher abundance of *Lactobacillus gasseri* (OTU_12) and *Bifidobacterium longum* (OTU_36) and neonates with UCLP displayed higher numbers of *Citrobacter freundii* (OTU_29) (Fig. 7d, f) compared to CPo at T1.

Discussion

Neonates born with OFC can present different cleft types affecting extraoral structures (lip) and/or intraoral (alveolus, hard and soft palate) [2] that can be associated with a complete or incomplete connection between nasal and oral cavity and an inefficient lip closure depending on the variety of cleft severities [2,4]. Since in neonates without OFC both cavities are parted by the palate and lip closure is adequate [2], differences in postnatal oral microbial composition can be expected with regard to the presence of OFC and with respect to cleft type and severity.

Considering oral microbiota after birth, oral genus frequencies showed no significant clustering into CLP and control neonates after birth. Yet, a trend to a distinct microbiome in CLP compared to control neonates can be seen. Accordingly,

a relatively homogeneous human microbiome without postnatal clustering was reported [28]. In all neonates, we found highest abundance of *Streptococcus* and high frequencies of *Staphylococcus*, *Gemella* and *Rothia* after birth, which is in accordance with a previous study in elderly neonates (2 months old) without CLP [29]. Similarly, it was shown that neonates with CLP (3 days and 1-2weeks old) presented relatively high abundance of the families *Streptococcaceae* and *Gemellaceae*[18] and high levels of *Staphylococcus* [*S.*] *aureus*, *S. epidermidis* and *Gemella haemolysans*[21]. However, the limitation of all those studies [18,21,28,29] is the missing comparison between CLP and control neonates. Regarding oral niches, we aimed to investigate two different niches (tongue and cheek) from the two expected metaniches ('S-T-HP' and 'C-U'), since young adults showed three distinct microbial metaniches ('P-GCF', 'S-T-HP' and 'C-U') [19] and 'P-GCF' will not be detectable in neonates without teeth. In contrast to adults, a microbial clustering into metaniches was not detectable in neonates after birth regarding beta diversity, Accordingly, Dominguez-Bello *et al.* [28] found no clustering into different body niches (skin, oral, nasopharyngeal, gut) after birth. While alpha diversity was not significantly different between both niches, some differences were seen since tongue samples presented significantly lower alpha diversity in CLP neonates compared to control neonates at both time points, while those differences were not seen in cheek samples. Those slight differences might be explained to the observed higher alpha diversity in niche T compared to niche C in adults [19].

Regarding postnatal microbiome maturation within the first weeks of life, our results presented a significant increase of alpha diversity in both groups. This is in accordance with a rising alpha diversity found in the oral microbiome from 6 to 24 months after birth [30] as well as during the first three years of life [29,31]. Within the first weeks of life, we detected an overall of 39 genera (117 OTUs) in neonates, which is partly in accordance with a previous study detecting 31 OTUs in the saliva of 2 months old neonates reaching an average of 84 OTUs until the age of 3 years using next generation sequencing [29]. Differences might be explainable by methodology. Notably, in young adults with oral health 300 OTUs were found [19] and, overall, 775 oral bacterial taxa were detected so far [14] indicating that the number of bacterial species raises with age. Moreover, we found a proportional increase of *anaerobic species* from T0 to T1 in both groups. In the control group, representing "physiological maturation" of oral microbiota, an increase of *stress-tolerant species* as well as an aggravation of *Haemophilus*, *Veillonella*, *Bergeyella*, *Lactobacillus*, *Actinomyces*, *Atopobium* and *Pseudomonas* was observed. Similarly, *Haemophilus parainfluenzae* presented high abundance in 2-month-old neonates without CLP [29].

Notably, the presence of orofacial clefts resulted in significant differences regarding composition and complexity of the oral microbiota compared to control neonates. Alpha diversity was significantly lower in CLP neonates compared to controls and lowest alpha diversity was found in neonates with high cleft severity. Interestingly, CLP neonates showed significantly lower microbial complexity of tongue samples. In healthy adults the close spatial proximity to the hard palate results in a distinct oral metaniche 'S-T-HP' formed by both sites and saliva samples [19]. Lower alpha diversity was also found in saliva samples from non-syndromic complete CLP (Ø 17 months) compared to controls [20]. The physiological increase of alpha diversity [29–31] is disturbed by changes in the oral (micro-)environment due to clefting (and subsequent PSIO) and this developmental alteration increases with increasing cleft severity. Further, a higher abundance of *Pseudomonas* and *gram-positive species* and lower abundance of *facultative anaerobic* and *gram-negative species* was seen in CLP neonates. *Pseudomonas aeruginosa* is the causative pathogen for different systemic diseases and was shown to coaggregate with oral key pathogens when colonizing the oral cavity [32–34]. *Gram-positive bacteria*, e.g. *S. aureus* have been linked to systemic infections [35], and present a risk for post-surgical inflammation [23].

With respect to orofacial clefts' influence on oral microbiome maturation within the first weeks of life, a clustering into neonates with CLP and control neonates was seen based on a dendrogram and were supported by MDS and CCA analyses where the CLP group formed a distinct cluster at T1. CLP neonates showed significantly higher values of *Enterobacteriaceae (Citrobacter, Enterobacter, Escherichia-Shigella, Klebsiella), Enterococcus, Bifidobacterium, Corynebacterium, Lactocaseibacillus, Staphylococcus, Acinetobacter and Lawsonella* compared to controls. In accordance with our findings, oral samples from neonates with CLP presented an increase of species belonging to the

genera *Klebsiella*, *Neisseria* and *Enterococcus* from 1–2 weeks until 8–18 weeks after birth [21]. Species of *Klebsiella* are *opportunistic pathogens* associated with infections and multi-drug-resistance [36,37] and species of *Enterococcus*, e.g., *E. faecalis*, are associated with perturbation of wound-healing[38,39]. Differences in oral microbiota were also seen in elderly CLP infants (17-months-old) compared to controls, e.g., lower amounts of *Lautropia* and *Bacillus* in CLP neonates [20]. Further, we found an elevation of *gram-negative* and *biofilm forming species*. Species of genera *Staphylococcus* and *Corynebacterium* (*gram-positive* and *facultative anaerobes* [40–42]) were shown to cause several bacterial infectious diseases in the human body [41]. Therefore, dysbiosis due to orofacial clefting may predispose for infections and wound-healing problems.

Considering CLP types, two patients with very low Severity Scores (CLo = 2, incomplete CPo = 4) clustered in the control group at T1 indicating that low cleft severity do not change the oral microbiome composition. Further, we found similarities between UCLP and BCLP neonates and differences between CPo and UCLP/BCLP neonates at both time points. Similarly, previous studies presented differences in microbiota composition between UCLP/BCLP and CPo neonates (1–5 months [21] and 6–7 months old [22]). Compared to BCLP/UCLP neonates (high cleft severity), CPo neonates showed at T0 higher levels of *anaerobic* and *facultative anaerobic species* as well of *Streptococcus* sp. At T1 we found higher abundances of *Klebsiella pneumoniae*, *Prevotella histicola*, *Streptococcus lactarius*, *Haemophilus parainfluenzae*, *Streptococcus parasanguinis*, *Chryseobacterium sp.*, *Corynebacterium kroppenstedtii*, *Neisseria sicca*, *Campylobacter concisus*, *Staphylococcus aureus*, *Prevotella histicola*, *Pseudomonas panacis* and *Veillonella atypica*. Notably, *Muribaculaceae*, *Escherichia*, *Staphylococcus* and *Lachnospiraceae* are dominant genera in the neonatal nasal microbiome [43].Enhanced levels of *Staphylococcus aureus* were correlated with increased infectious lethality in neonates [44] and high levels of *Prevotella* [43] (*obligate anaerobes* [45]) were associated with bronchopulmonary dysplasia in preterms [43]. Moreover, BCLP/UCLP neonates (high cleft severity) presented significantly lower levels of *gram-negative* and significantly higher levels of *gram-positive* species at T0. Notably, *gram-positive anaerobes* were associated with infections and wound healing disorders [46–48], e.g., *Staphylococcus aureus* [47,48], *Enterococcus faecalis* [47] and *Propionibacteriaceae* [49,50]. Compared to controls, BCLP neonates presented higher numbers of *Lactobacillus gasseri* and *Bifidobacterium longum* compared to CPo at T1. Interestingly, the skin microbiota contains several beneficial species, e.g. *Lactobacillus* [51], an *oxygen-tolerant anaerobe*, which is also used as probiotic supplement to promote oral health [52,53]. UCLP neonates presented significantly higher abundance of *Citrobacter freundii* compared to CPo at T1, which was associated with infections and multidrug resistance [54]. Dissimilarities between CPo and UCLP/BCLP neonates can be explained by the different phenotypes: CPo are characterized by an insufficient separation of nasal and oral cavity, however, without affection of extraoral structures, while clefting in UCLP and BCLP affects extraoral and intraoral anatomical structures [2,4]. Therefore, the oral microbiota of both CPo and UCLP/BCLP neonates might be composed by oral and nasal microbiota components, while in UCLP and BCLP the insufficient lip closure might also lead to a mixture with skin microbiota. Further, different oxygen levels in the oral cavity in CPo compared to UCLP/BCLP might impact the growth of *anaerobic species* in CPo neonates. Therefore, risk factors for wound healing disorders might also differ between cleft phenotypes with an increased risk in UCLP/BCLP neonates due to the mixture with skin microbiota and in CPo neonates due to higher levels of *anaerobic species*.

The limitation of this study is that the exogeneous factors birth mode, antibiotic intake intrapartum (mothers with C-section) and different nutrition modes due to feeding issues in the CLP group could not be excluded. Previous studies detected an impact of birth mode [25] and intrapartum antibiotic therapy [18] on the oral microbiome composition. However, different birth modes as well as antibiotic intake intrapartum were distributed equally in both groups of our study and we detected no differences between vaginal and C-section (in most cases positively correlated with antibiotic intake intrapartum) born neonates in the CLP group. Hence, differences found between the control and CLP groups are not biased due to the different birth modes and due to antibiotic intake intrapartum. With regard to nutrition, the control group

in our study was exclusively breast-fed, whereas the CLP group showed mixed nutrition modes that can't be excluded due to the well-known feeding issues in CLP neonates after birth. However, different nutrition modes were distributed equally with regard to cleft phenotype and severity, e.g., breast-milk bottle-feeding was found in neonates with CPo (LKG-005) and UCLP neonates (LKG-007, LKG-011) and postnatal tube feeding was seen in CPo (LKG-002, LKG-016) and BCLP neonates (LKG-10, LKG-012) (Table 1). Subgroup analyses showed a trend towards higher levels of *facultative anaerobic species* in oral microbiota of breast milk (bottle) fed (breast or bottle-feeding) neonates compared to formula-bottle-fed (partially or complete artificial baby food) neonates (d.n.s.), which is in accordance to previous studies detecting higher abundance of *Lactobacillus* and *Bifidobacteriae* (*gram-positive* and *facultative anaerobic species* [55,56] used as probiotics in preventive treatments [52,53,56]) in the gut microbiome of 4-months old infants. Taken together, there might be a positive effect of breast-milk (bottle) feeding with regard to oral microbiome maturation, however, larger sample sizes are needed in future studies.

In conclusion, the present study revealed that the postnatal 'core' oral microbiome is characterized by an evenly distributed *Streptococcus* spp.-dominated microbial community with high abundance of *Staphylococcus*, *Gemella* and *Rothia* and that the 'symbiotic' early life oral maturation process is characterized by an increase of alpha diversity, an elevation of distinct genera (*Haemophilus*, *Veillonella*, *Bergeyella*, *Lactobacillus*, *Actinomyces*, *Atopobium*, *Pseudomonas*) and phenotypes (*anaerobe* and *stress-tolerant species*) associated with health. While distinct oral metaniches [19] were not seen in neonates, there is some evidence, that the formation of metaniches starts after birth with an increased alpha diversity in tongue samples. However, physiological maturation of the microbiota as exemplified by an increase of alpha diversity is disturbed by clefting and this developmental alteration increases with increasing cleft severities. Further, higher abundance of *gram-positive species* found in CLP neonates might explain the risk for wound healing disorders post-surgical inflammation. OFC impact early life oral microbiota development towards a dysbiosis of oral microbiota with increased levels of *gram-positive and 'dysbiotic' species* [*Enterobacteriaceae* (*Citrobacter*, *Enterobacter*, *Escherichia-Shigella, Klebsiella*), *Enterococcus*, *Bifidobacterium*, *Corynebacterium*, *Lactocaseibacillus*, *Staphylococcus*, *Acinetobacter and Lawsonella*] presenting a potential risk for oral inflammation [38,39], wound healing disorders [23] and dysbiotic inflammatory diseases later in life [24]. Neonates with cleft affecting the palate showed a mixture with nasal microbiota, while neonates with clefts affecting also extraoral structures presented a mixture with skin microbiota indicating that the involvement of intraoral and extraoral structures by clefting plays a major role.

The knowledge of age-related changes of oral microbiota after birth is necessary for a better understanding of the regular maturation process of oral microbiota as well as for an early detection of pathological developments. Future studies are necessary to improve our knowledge about the formation of oral niches and microbiota maturation from birth until adulthood. Our findings about oral microbiota adaptations in neonates with orofacial clefts, especially in neonates with high cleft severity, is clinically relevant. We suppose that postnatal oral dysbiosis not only presents a risk factors for wound healing disorders and failure of surgical cleft closure within the first year of life, but might causative for the predisposition to oral diseases later in life. Longitudinal studies investigating oral microbiota alterations prior to and after lip (4–6 months of age) and palate surgery (10–12 months of age) are needed to correlate increased oral pathogens in neonates with OFC to wound healing disorders and to identify the most pathogen species. In the future, a dysbiosis panel for neonates with OFC might present a helpful tool, e.g., in case of detection of severe dysbiosis prior to cleft surgery it might be helpful to postpone the surgery for 2–4 weeks to reduce the risk for failure of surgery by reduction of dysbiosis. Novel therapeutic approaches to inhibit the postnatal development of oral dysbiosis should be focus of future studies, e.g., postnatal supplementation of probiotics might be a promising approach since beneficial effects were not only seen in the oral health field [52,53,57] but also in treatment of preterm infants [58] and neonatal diseases[59], e.g., necrotizing enterocolitis [60] and infantile colic [61]. Preliminary studies are needed to identify which specific probiotic strain might be most beneficial in neonates with OFC. Bottle feeding with breast-milk (instead of artificial baby food) might depict an

adjuvant strategy and should be especially recommended to mothers of neonates with OFC due to the potentially beneficial impact.

To conclude, the detection of postnatal oral dysbiosis in neonates with orofacial clefts, especially in those with high cleft severity, has a great clinical importance. The consequence of our results might arrogate a standard monitoring of oral microbiota in neonates with OFC (at least in with higher severity) within the first year of life to identify an increase of dysbiosis-associated pathogens, e.g., *Enterobacteriaceae (Citrobacter, Enterobacter, Escherichia-Shigella, Klebsiella).* Further studies are essential to define a dysbiosis panel prior to cleft surgery and to investigate preventive strategies to inhibit oral dysbiosis after birth and before surgical interventions.

Methods

This study was designed as a prospective, exploratory observational clinical trial and has been approved by t 12 he local ethics committee of the Friedrich-Alexander-University Erlangen-Nürnberg (Krankenhausstraße 12, 91054 Erlangen, Vote number: 168_20 B, 28.04.2020) prior to the beginning of the study. The trial was performed in accordance to the declaration of Helsinki. A total of 107 patients were recruited following predefined inclusion criteria: 1) Neonates with non-syndromic orofacial cleft (first consultation at the Department of Orthodontics and Orofacial Orthopedics within the first days and weeks of life) OR neonates without orofacial cleft (born in the Department of Gynecology and Obstetrics Erlangen with ongoing regular consultations in local pediatric practice) and 2) written informed consent by the parents and/or legal guardians. Exclusion criteria were defined as: 1) Neonates with syndromic cleft lip and palate, 2) Preterm birth (< 37 weeks gestational age), 3) neonates with underweight at birth (< 2500 g), 4) neonates with systemic and metabolic diseases with potential influence on inflammation and microbiota, 5) revoked written informed consent by the parents and/or legal guardians. Two informed consent forms for participation in the trial and for utilization of saliva and tissue samples, data protection sheets and information material explaining the study in adequate language were provided. Written informed consent forms and data protection sheets by the parents and/or legal guardians were mandatory for enrollment in the trial. Moreover, written questionnaires were given to the parents and/or legal guardian to collect information about neonates' clinical parameters including weight and height at birth, nutrition protocol, intake of antibiotics and/or supplements as well as to collect information about the mother including information type of birth (vaginal, caesarian), premature rupture of membranes (PROM) and intake of antibiotics (Table 1).

Recruitment phase

After eligibility screening, a total of 40 study participants were enrolled in this study and divided into two groups (Fig. 8). The study group, neonates (n = 18) with orofacial clefts, was recruited at the Department of Orthodontics and Orofacial Orthopedics, Universitätsklinikum Erlangen, Friedrich-Alexander Universität (FAU) Erlangen-Nürnberg. The control group, neonates without orofacial clefts (n = 22), was recruited at the Department of Gynaecology and Obstetrics, Universitätsklinikum Erlangen, FAU Erlangen-Nürnberg. Dropouts were registered due to the following reasons: 1) Meeting the exclusion criteria during the course of the study, e.g., diagnosis of a syndrome or acute systemic or metabolic disease (CLP group: n = 1, control group: n = 2), 2) revoke of consent by the parents and/or legal guardians (CLP group: n = 1, control group: n = 3), or 3) Failure to appear to the consultation and study appointments (CLP group: n = 1, control group: n = 0) (Fig. 8). In total, the dropout rate was 15% (n = 8 in total) with a final sample size of 15 study participants in the study group and 17 study participants in the control group (Fig. 8).

Sample collection

After obtaining informed consent, 158 samples were collected from 40 study participants at two time points (T0, T1) from two different oral niches from each study participant from June 2020 to June 2021. Due to drop-outs, 32 samples

were excluded from further analyses (Fig. 8). Therefore, the final sample size accounted to 126 samples from 32 study participants (Fig. 8). The oral niches tongue (T) and cheek (C) were chosen as representatives of two of the three previously detected metaniches [19]. The third metaniche (gingival crevicular fluid and plaque) [19] is not expected in neonates since tooth eruption and congenital eruption cysts rarely occur in neonates [62]. Soft tissue samples were collected using sterile swabs patting over the area T (middle and anterior part) and C (right side) for about 10 seconds and parents were instructed not to feed the neonate up to two to three hours before sample collection. Regarding the cleft lip and palate (CLP) group, the first time point (T0) was defined as the first consultation at the Department of Orthodontics and Orofacial Orthopedics, which is a regular appointment for clinical investigation (T0: median 3d CLP group). On this appointment, orthodontists specialized in the treatment of newborns with orofacial cleft investigate the newborn, make a diagnosis and decide whether presurgical treatment with a palate plate is needed. In case of treatment need, neonates have regular appointments every 4–6 weeks. The second time point (T1) was defined as a regular control appointment for the CLP group 4–5 weeks after birth (T1: median 32d CLP group). Considering the control group, the first time point (T0) was defined as the second (‘U2’) routine investigation for children after birth (called ‘U’ investigations) performed in the Department of Gynecology and Obstetrics (median Ø2 d control group). The second time point (T1) was defined as the third (‘U3’) routine investigation at local pediatricians for the control group after birth (T1: median 31d control group). Since the first consultation in the CLP group earliest takes place when transport of neonates is possible, the median age for the CLP was 1 day elder than in the control group at T0 ($p = 0.046$). However, differences at T1 were not detectable (Table 2). Samples were collected in sterile tubes and stored on dry ice within seconds after sample collection. In general, samples were directly frozen and stored at -80°C (CryoCube unit) in the research laboratory of the Department of Orthodontics and Orofacial Orthopedics. For interim storage, samples were frozen at -20°C for a maximum of 5 days under maintenance of an uninterrupted cold chain. Further processing and DNA isolation of samples was performed at the research laboratory of the Department of Orthodontics and Orofacial Orthopedics. Microbiota analyses was conducted in the Institute of Clinical Microbiology, Immunology and Hygiene.

Table 2
Statistical comparison of study and control group characteristics

Characteristics		CLP group (n = 15)	Ctrl group (n = 17)	p-value
Age	T0 (d) *	3 (2-7.25)	2 (2–3)	0.046[a]
	T1 (d) *	32 (24.75-38)	31 (24-35.5)	0.717[a]
Gender	f, n (%)	5 (33)	7 (41)	0.789[b]
	m, n (%)	10 (67)	10 (59)	0.561[b]
Weight	T0 (g) *	3120 (2920–3328)	3480 (3165–3870)	0.024[a]
Height	T0 (g) *	51 (49.75-51)	53 (50–54)	0.051[a]
Type of birth	v, n (%)	11 (73)	10 (59)	0.659[b]
	c, n (%)	4 (27)	7 (41)	0.485[b]
Antibiotics	noAB	9 (60)	10 (59)	0.638[b]
	ABM	5 (33)	7 (41)	0.718[b]
	ABN	1 (7)	0 (0)	0.287[b]
Nutrition [1]	0	2 (13)	17 (100)	0.001[b]
	1	3 (20)	0	0.065[b]
	2	4 (27)	0	0.033[b]
	3	2 (13)	0	0.132[b]
	4	4 (27)	0	0.033[b]

d = days, g = grams, cm = centimeter, f = female, m = male, n = number; v = vaginal, c = caesarian, noAB = no antibiotic intake of neonates or mother before birth, ABM = antibiotic intake mother intrapartum, ABN = antibiotic intake neonate after birth, T0 = time point T0 after birth, T1 = Time point T1 4–5 weeks after birth

[1] 0 = breastfeeding, 1 = bottle feeding breast milk, 2 = bottle feeding partly breast milk, partly artificial baby food, 3 = bottle feeding artificial baby food, 4 = postnatal tube feeding for < 1 week (= T0);

* median (IQR); [a] Mann-Whitney Test; [b] chi-square Test;

Study population

Characteristics of the CLP and control group were collected (Table 1) and compared statistically (Table 2). No differences were seen with regard to gender, type of birth or intake of antibiotics (Table 2). However, statistical differences were seen regarding birth weight: The CLP group showed significantly lower median weight at T0 (3120 g at T0) compared to the control group with a median weight (3480 g at T0) in conformity with the European average [63] (Table 2). Compatible with the lower birth weight at T0, the CLP group presented a slightly lower median height at T0 (51cm) (Table 2). Moreover, significant differences were seen with regard to nutrition, which is consistent with reported feeding difficulties in the CLP group [10,11] compared to 100% breast feeding in the control group (Table 2). To insure proper daily food intake, different nutrition methods like bottle feeding with Haberman feeder [12] and in severe cases supplementary use of nasogastric tube

feeding is necessary [13] (Table 2). Further, neonates with orofacial cleft showed specific characteristics with regard to cleft type, cleft severity and need for treatment (Table 1). The final study group comprised neonates with BCLP (n = 5), UCLP (n = 4), CPo (n = 5) and CLo (n = 1) (Table 1). The LAHSHAL classification scheme by Kriens *et al*. [5] uses letters to describe the anatomical parts affected by cleft in the following sequence: right lip, right alveolus, right hard palate, soft palate, left hard palate, left alveolus, left lip. Capital letters represent complete clefting of the affected anatomical part (L = lip, A = alveolus, H = hard palate, S = soft palate), while small letters (l, a, h, s) depict incomplete clefting of the affected structure [5]. Anatomical parts that are not affected by clefting are described with a minus sign, minimal clefting with an asterisk and skin band with a plus next to the letter [5], e.g., the LASHSAL code describes complete bilateral cleft lip palate, LAHS— describes complete unilateral cleft lip palate on the right side and –hSh– describes cleft palate only with incomplete cleft of the hard palate and complete cleft of the soft palate. In order to calculate the severity of the clefting (LAHSHAL Severity), we developed a “LAHSHAL Severity Score”, which was the sum of the numbers of the LAHSHAL Severity: 0 = not affected, 1 = incompletely affected, 2 = completely affected (Table 1). Hence, the most severe form of clefting, the complete bilaterial cleft lip and palate (BCLP LAHSHAL Code: LAHSHAL), is described with 2222222 summing up to LAHSHAL Severity Score 14 (Table 1). While neonates with severe cleft severity are in need of treatment with palate plates (pAM) [64], neonates with minor cleft severity sometimes present no feeding problems and are therefore not in need of treatment and require less appointments (Table 1). Hence, two neonates with incomplete CPo (LKG_002 and LKG_004) required only one appointment and only one time point was collected (LKG_002 only T1: late first consultation with no treatment need; LKG_004 only T0: no need for further treatment).

Microbiome analyses

DNA isolation, amplification of the V1-V3 region of the 16S rDNA, sequencing and raw read processing was carried out as described before [19]. Sequencing data is available through the National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA) under BioProject: *Number to be added*. The following samples were excluded for microbial analyses: One sample (LKG-017-T1-T) was ~ 20x oversampled, samples LKG-005-T0-C, LKG-005-T1-C, LKG-008-T1-C, LKG-016-T1-T, LKGc-016-T0-C and LKGc-016-T0-T were excluded due to very low read counts. After removal of these samples the read median was 35,188. For OTU-based classification with the USEARCH v11 algorithm [65] sequences were re-multiplexed using a Perl script (I. Lagkouvardos, available from www.imngs.org) and submitted to the IMNGS server [66]. Taxonomic classification of the identified OTUs was done using the SILVA alignment, classification and tree service [67] with SILVA database release 138.1 and NCBI nucleotide BLAST [68]. Further analyses were done within R v4.2.0 [69] by importing the modified OTU table, the SILVA phylogenetic tree and metadata using the package phyloseq [70]. Phyloseq functions were used to filter the data, calculate and visualize diversitiy and to perform ordination. For ordination we used log-transformed counts to stabilize variance and calculated distances based on the generalized UniFrac algorithm (GUniFrac package for R). Differential abundance analysis was done using EdgeR [70], limma [71] and voom [72] with ggplot2 [73] for visualization. BugBase [26] was used to estimate the proportion of "high level phenotypes" such as *gram-positive*, *gram-negative*, *anaerobes*, *aerobes*, *biofilm-formers*, *stress tolerant strains* or *bacteria with mobile elements*. For this analysis, the OTU table and -sequences were imported in QIIME 2 v2021.11 [74] and taxonomic classification was done with VSEARCH [75] against the GreenGenes v13.8 16S rRNA database [76] with 97% similarity threshold. The data was exported as biom (v1) file and finally submitted to the BugBase server (https://bugbase.cs.umn.edu/).

Statistics

The statistical analyses of the oral microbiome was done with Microsoft Excel 2016 (Microsoft, Redmond, WA, USA), R v4.2.0 with the packages vegan [69] and pairwiseAdonis (https://github.com/pmartinezarbizu/pairwiseAdonis). Statistical analysis of beta diversity was done with permutational multivariate analysis of variance (PERMANOVA) as implemented in the adonis2 function of the vegan package. Similar dispersion of the analyzed groups was tested with betadisper and permutest functions (vegan) and subsequent Tukey’s HSD test. The statistical investigation of the study population was

performed with Microsoft Excel 2016 (Microsoft, Redmond, WA, USA) and GraphPad Prism 9 statistical software (GraphPad Software, San Diego, CA, USA). The data sets were analyzed by using the Mann-Whitney U-test and chi-square Test. Differences were considered significant with p-values < 0.05.

Declarations

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Author Contributions

Conceptualization, C.L.S. and L.G.; methodology, C.L.S., M.W. and R.G.G.; formal analysis, C.L.S.; recruitment and sample collection C.L.S., K.S., M. T. and U.C.; clinical examination C.L.S. and K.S.; resources and clinical examination control group A.H., P.M., M.S., M.W.B.; characteristics of the study population C.L.S.; microbiome analyses and visualization R.G.G.; concept of group/subgroup analyses, discussion and interpretation of microbiome analyses C.L.S.; writing—original draft preparation C.L.S.; writing—review R.G.G., M.W., I.S., MN.W. C.B. and L.G.; project administration C.L.S. and K.S.; supervision L.G.. All authors have read and agreed to the published version of the manuscript.

Statement of Ethics

This study has been approved by the local ethics of the Friedrich-Alexander-University Erlangen-Nürnberg (Krankenhausstraße 12, 91054 Erlangen, Vote number: 168_20 B, 28.04.2020) prior to the beginning of the study.

Competing Interest

The authors declare no conflict of interest. Dr. Corinna Lesley Seidel declares that she has received internal financial funding (ELAN) by the IZFK (Interdisziplinäres Zentrum für Klinische Forschung) by the Friedrich-Alexander Universität (FAU) Erlangen-Nürnberg (Grant holder: Corinna Lesley Seidel, Project number: P086).

Data availability

Participants of the study will be informed about the findings of the trial, but only if desired. Raw sequencing data together with non-personal metadata is available through a publicly accessible database (BioProject: xxxxx). Upon reasonable request and after approval by data protection commissioner, further de-identified metadata may be made available by the corresponding author once the trial is completed.

Abbreviations

A	Alveolous (LAHSHAL code)
ABM	antibiotic intake mother intrapartum
BCLP	Bilateral cleft lip and cleft palate
c	caesarian
CCA	constrained correspondence analysis
CLo	Cleft lip only
CLP	Cleft lip and cleft palate
cm	centimeter
CPo	Cleft palate only
CTRL	control
d	days
d.n.s.	data not shown
f	female
g	grams
H	Hard Palate (LAHSHAL code)
HP	Hard Palate
L	Lip (LAHSHAL code)
m	male
MDS	multidimensional scaling
n	number
NAM	Nasoalveolar Molding
NGS	next generation sequencing
noABM	no antibiotic intake by mother intrapartum
OFC	Orofacial clefts
p	Cleft of the primary palate
pAM	Passive Alveolar Molding
ps	cleft of the primary and secondary palate
s	Cleft of the secondary palate
S	Soft Palate (LAHSHAL code)
T	Tongue
T0	Time Point T0 after birth
T1	Time point T1 4-5 weeks after birth
UCLP	Unilateral cleft lip and cleft palate
v	vaginal

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Figures

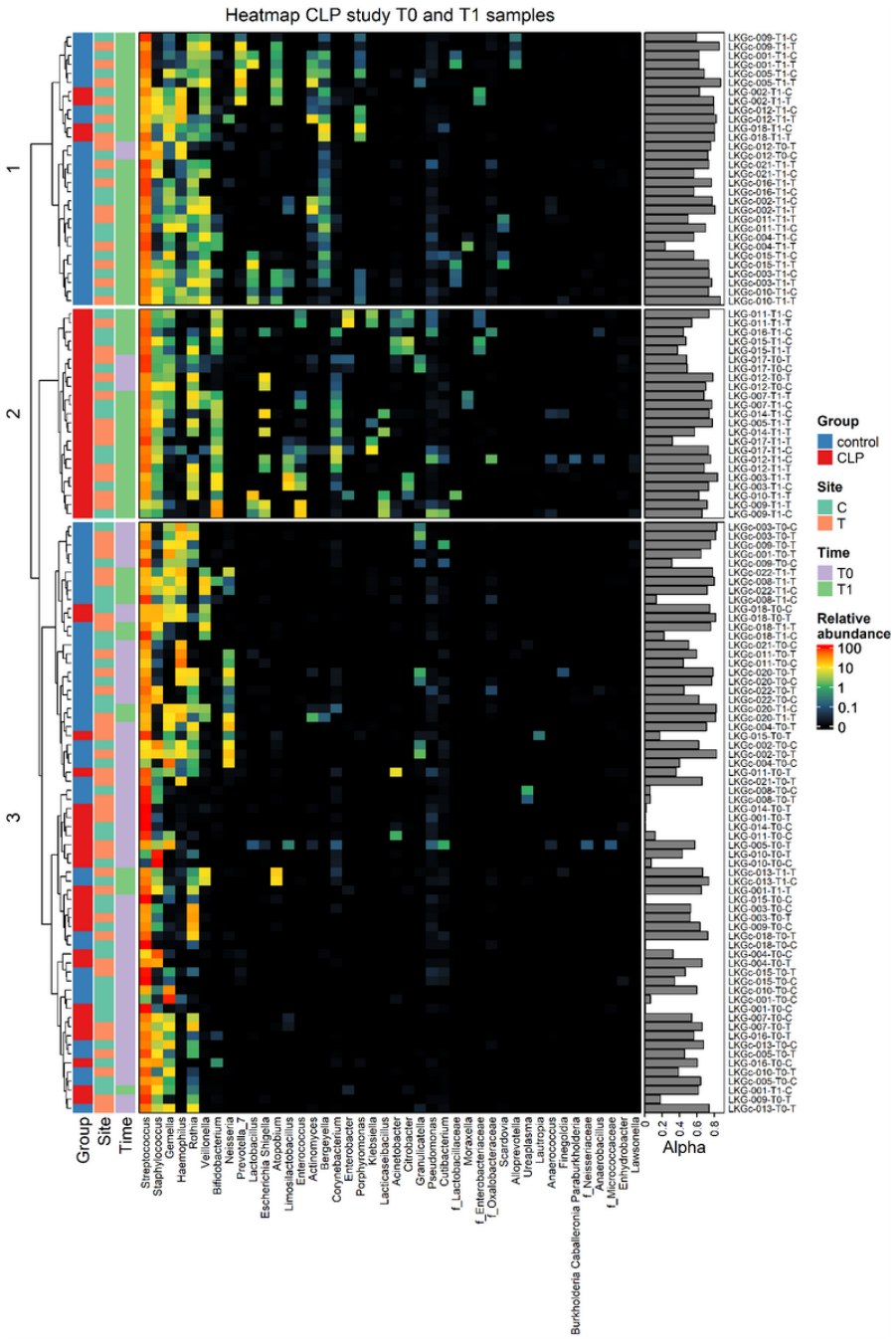

Figure 1

Dendrogram and genus frequencies plot. A dendrogram based on generalized UniFrac distances is given for each individual sample (excluded samples see Material & Methods) collected from two groups (red: cleft lip palate ("LKG"/CLP) group; blue: control ("LKGc") group), two niches (orange: tongue T; turquoise: cheek C) and two time points (purple: T0, green: T1). A heatmap representing relative abundances (black = no abundance; red = highest abundance) of

microbial genera found in individual samples. Samples are ordered vertically according to the dendrogram, while microbial genera are presented horizontally with left to right decreasing mean relative abundance in all samples. The Simpson diversity index representing alpha diversity (Alpha) is shown on the right side for each sample with sample identifier next to it.

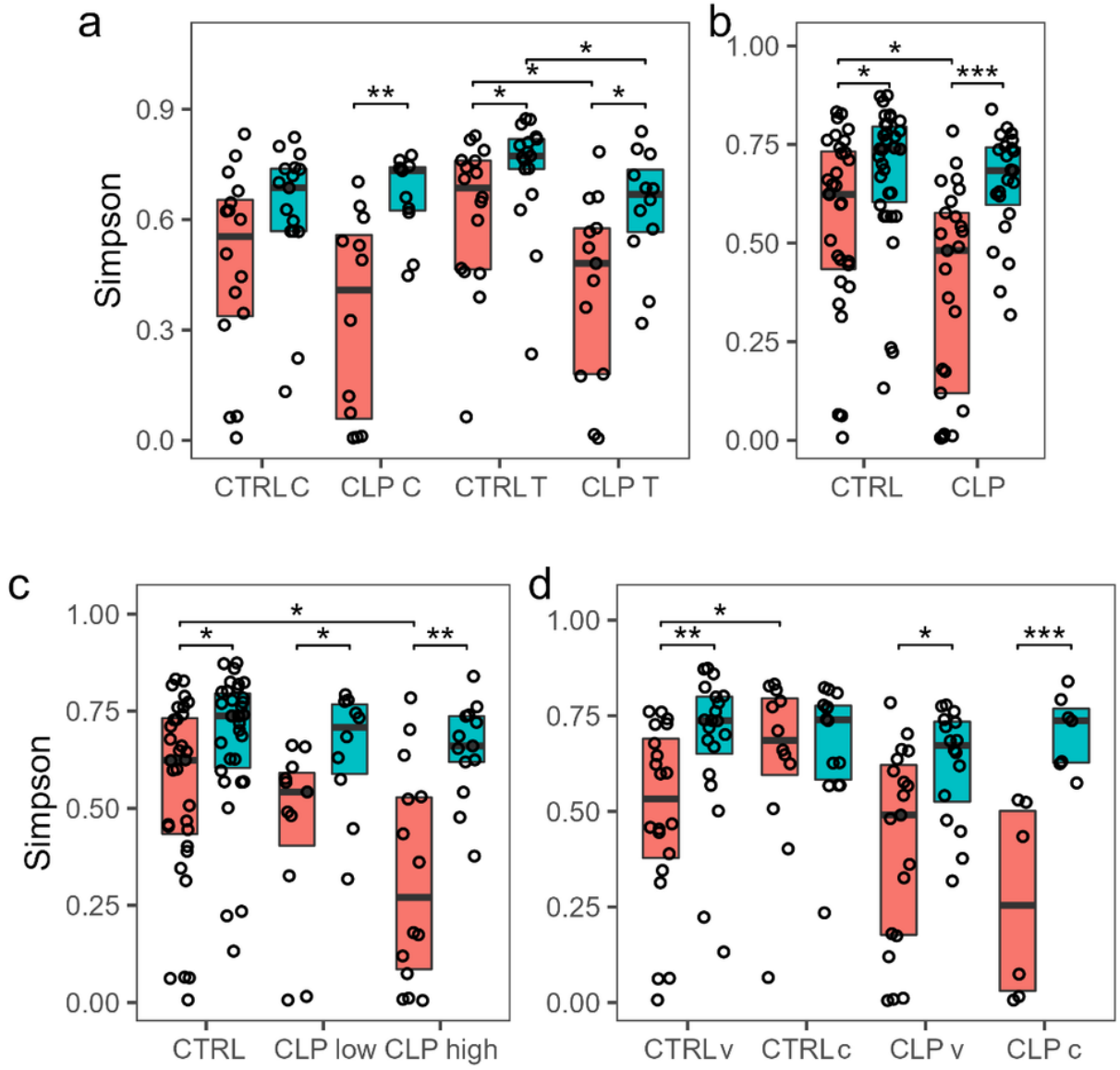

Figure 2

Alpha Diversity. Box and scatter plots of Simpson diversity is given for both time points T0 and T1 (orange = T0; turquoise = T1) for (a) cleft lip palate (CLP) vs. control (CTRL), tongue (T) vs. cheek (C); (b) CLP vs. CTRL; (c) CTRL vs. CLP with low Severity score 2-7 (CLPlow) vs. CLP with high Severity score 8-14 (CLPhigh); (d) CLP vs. control CTRL considering different type of birth vaginal (v) vs. caesarian birth (c). T1). Simpson diversity of individual samples was calculated based on OTU counts (dots). Boxes show 25th percentile, median and 75th percentile. Wilcox tests were used to calculate pairwise comparison statistics as indicated. Differences were considered statistically significant for $p<0.05$ (*), $p<0.01$ (**), $p<0.001$ (***).

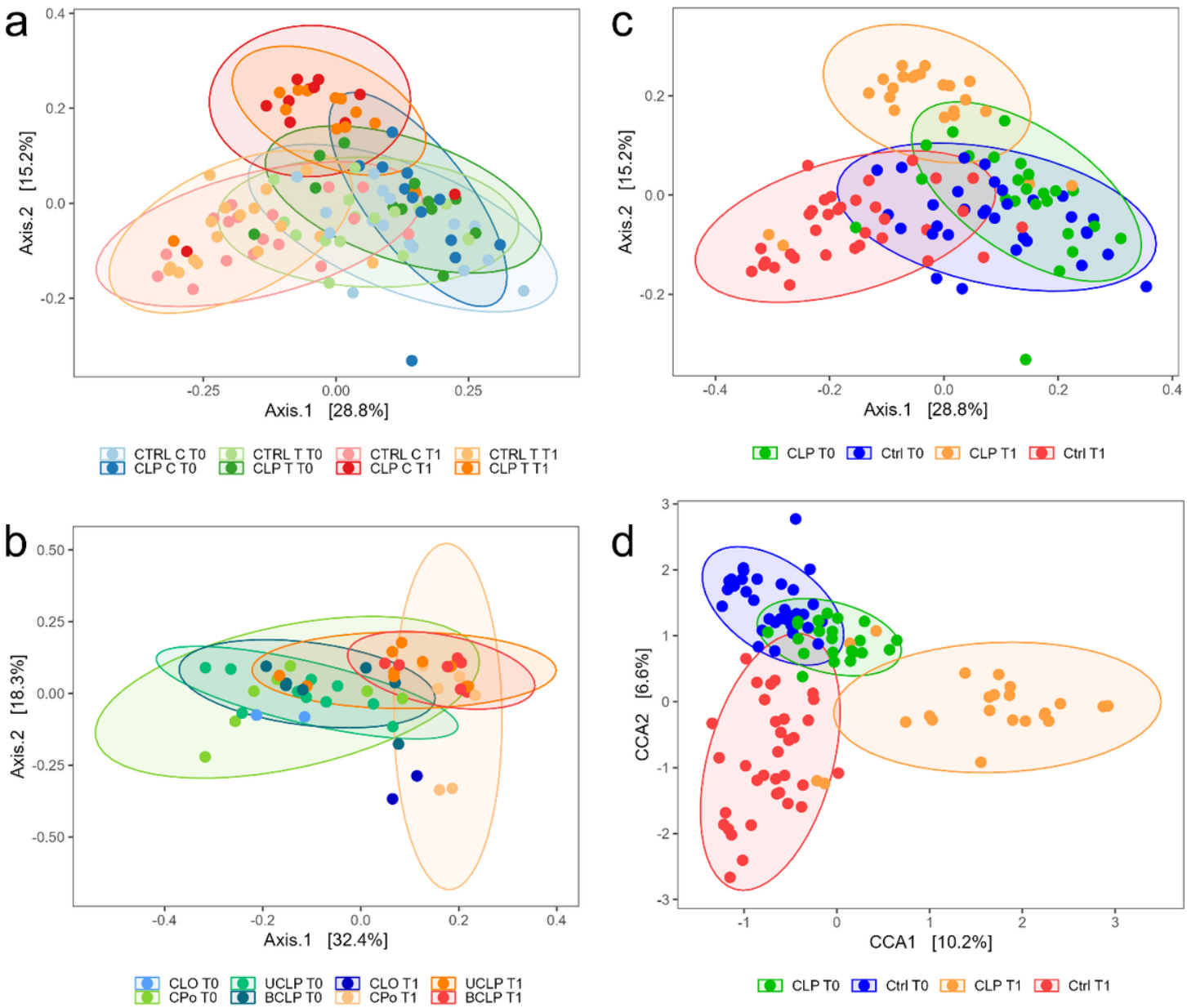

Figure 3

Beta Diversity. Multidimensional scaling (MDS) plots based on generalized UniFrac distances of log-transformed genus counts are shown for (a) cleft lip palate (CLP) vs. control (Ctrl), tongue (T) vs. cheek (C) and time points T0 vs. T1; (b) Cleft Lip only (CLo) vs. Cleft Palate only (CPo), unilateral cleft lip palate (UCLP), bilateral cleft lip palate (BCLP) and T0 vs. T1 and (c) CLP vs. Ctrl and T0 vs. T1. In (d) a Canonical Correspondence Analysis (CCA) of log-transformed genus counts constrained to group (CLP, Ctrl) and time point (T0, T1) is depicted.

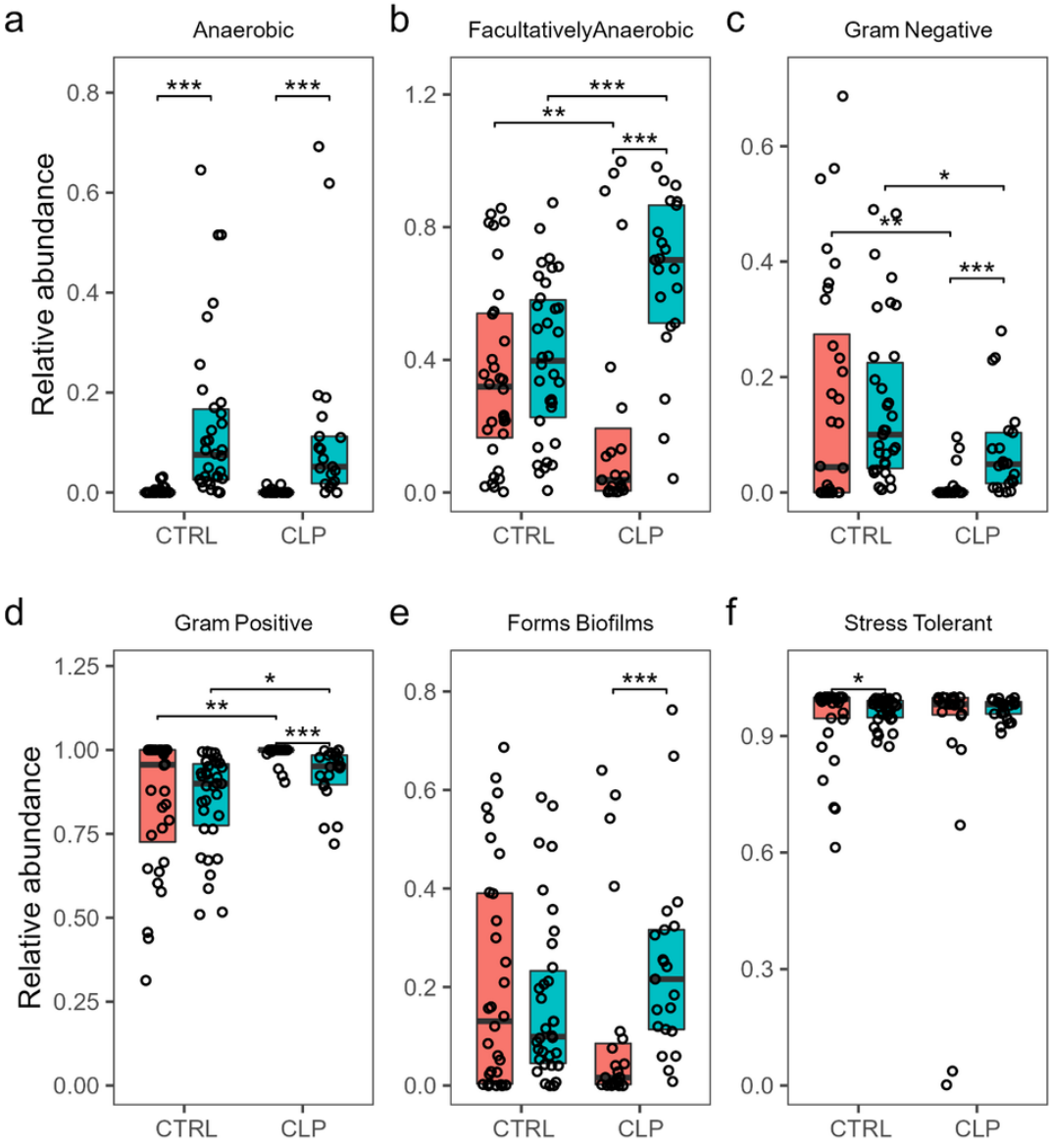

Figure 4

High level phenotype analysis. Relative abundance is given for cleft lip palate (CLP) vs. control (CTRL) and time points T0 (orange colour) vs. T1 (turquoise colour) regarding (a) aerobic, (b) facultative anaerobic, (c) anaerobic, (c) gram-negative, (d) gram-positive, (e) biofilm forming and (f) stress tolerant species. Wilcox tests were used to calculate pairwise comparison statistics as indicated. Differences were considered statistically significant for $p<0.05$ (*), $p<0.01$ (**), $p<0.001$ (***).

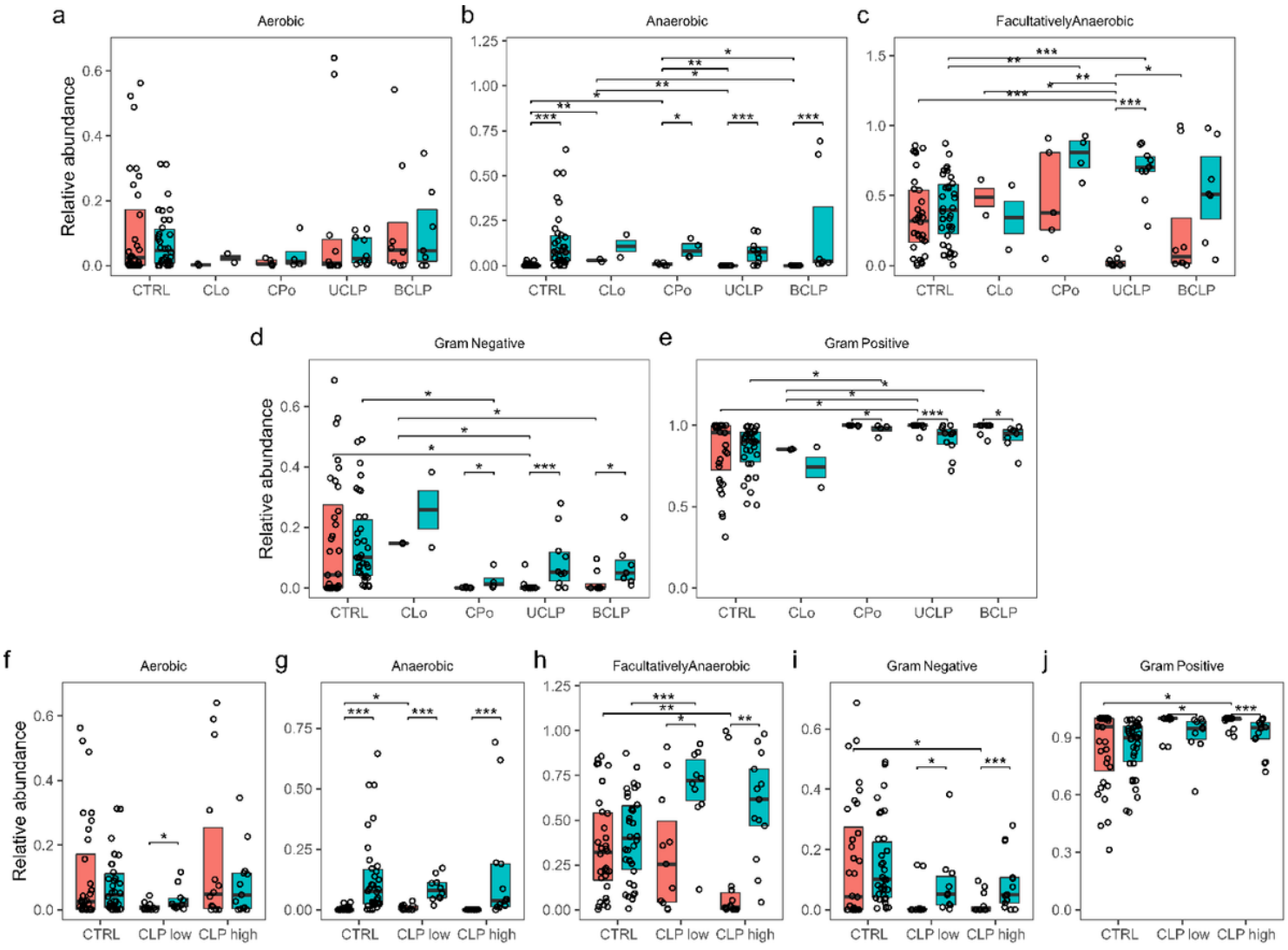

Figure 5

High level phenotype analysis regarding cleft phenotype and severity. Relative abundance is given for different cleft phenotypes Cleft Lip only (CLo), Cleft Palate only (CPo), unilateral cleft lip palate (UCLP), bilateral cleft lip palate (BCLP) and time points T0 (orange colour) vs. T1 (turquoise colour) regarding (a) aerobic, (b) anaerobic, (c) facultatively anaerobic, (d) gram-negative, (e) gram-positive species. Relative abundance is given for CLP with low Severity score 2-7 (CLPlow) vs. CLP with high Severity score 8-14 (CLPhigh) and time points T0 (orange colour) vs. T1 (turquoise colour) regarding (f) aerobic, (g) anaerobic, (h) facultatively anaerobic, (i) gram-negative, (j) gram-positive species. Wilcox tests were used to calculate pairwise comparison statistics as indicated. Differences were considered statistically significant for $p<0.05$ (*), $p<0.01$ (**), $p<0.001$ (***).

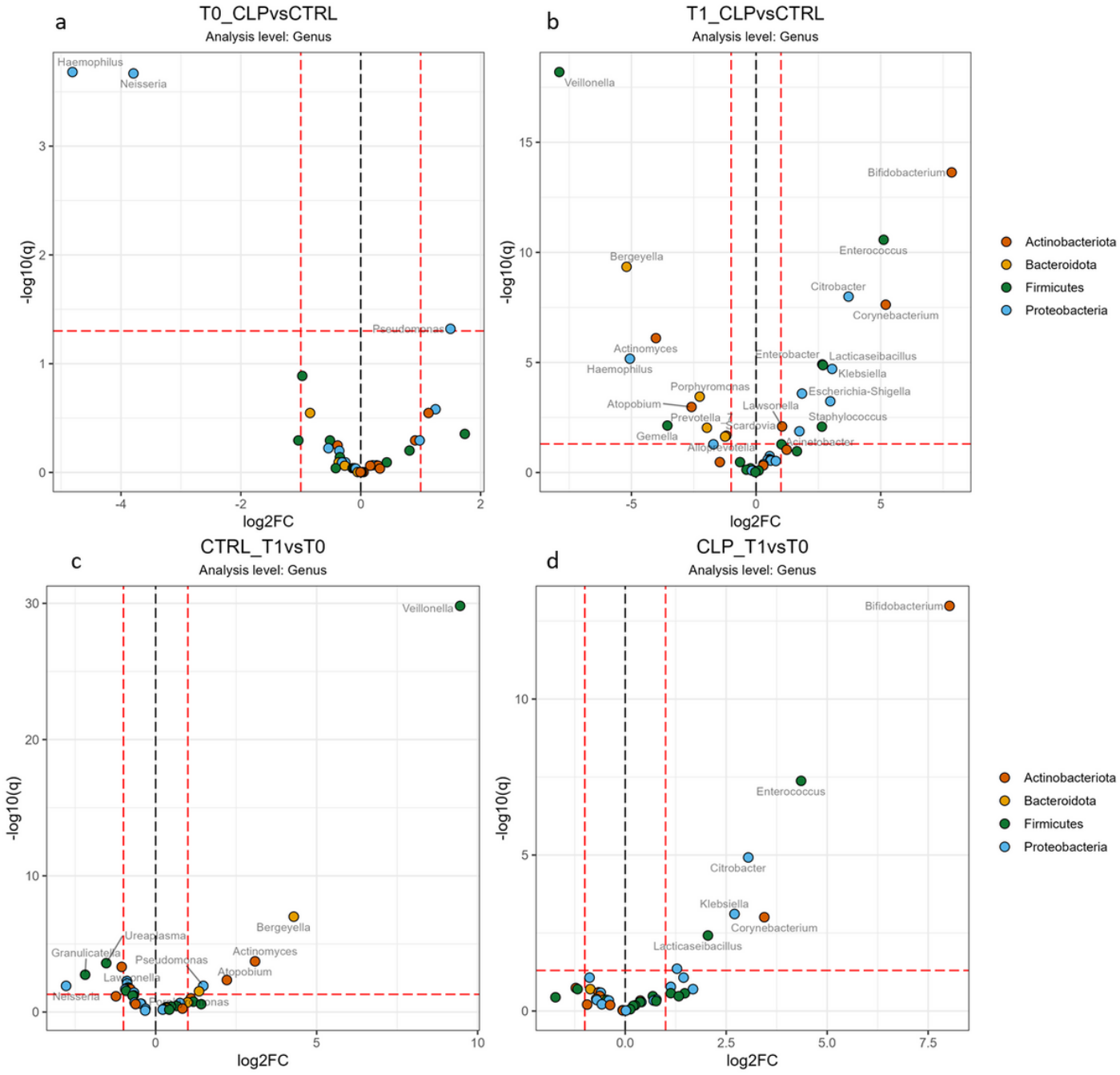

Figure 6

Differential analysis of Beta-diversity. Volcano plots on genus level is given for a) the cleft lip palate (CLP) vs. control (CTRL) group at T0 and (b) CLP vs. CTRL at T1; d) time point T0 vs. T1 for CTRL group and d) T0 vs. T1 for CLP group. Genus are given next to colored dots, which follow a color scheme to visualize the corresponding phyla (orange dots = Actinobacteriota; yellow dots = Bacteroidota; light green dots = Campylobacterota; dark green dots = Firmicutes; blue dots = Proteobacteria) and dashed lines indicate significant differences (>2-fold, adj. p (q) < 0.05) between the comparators.

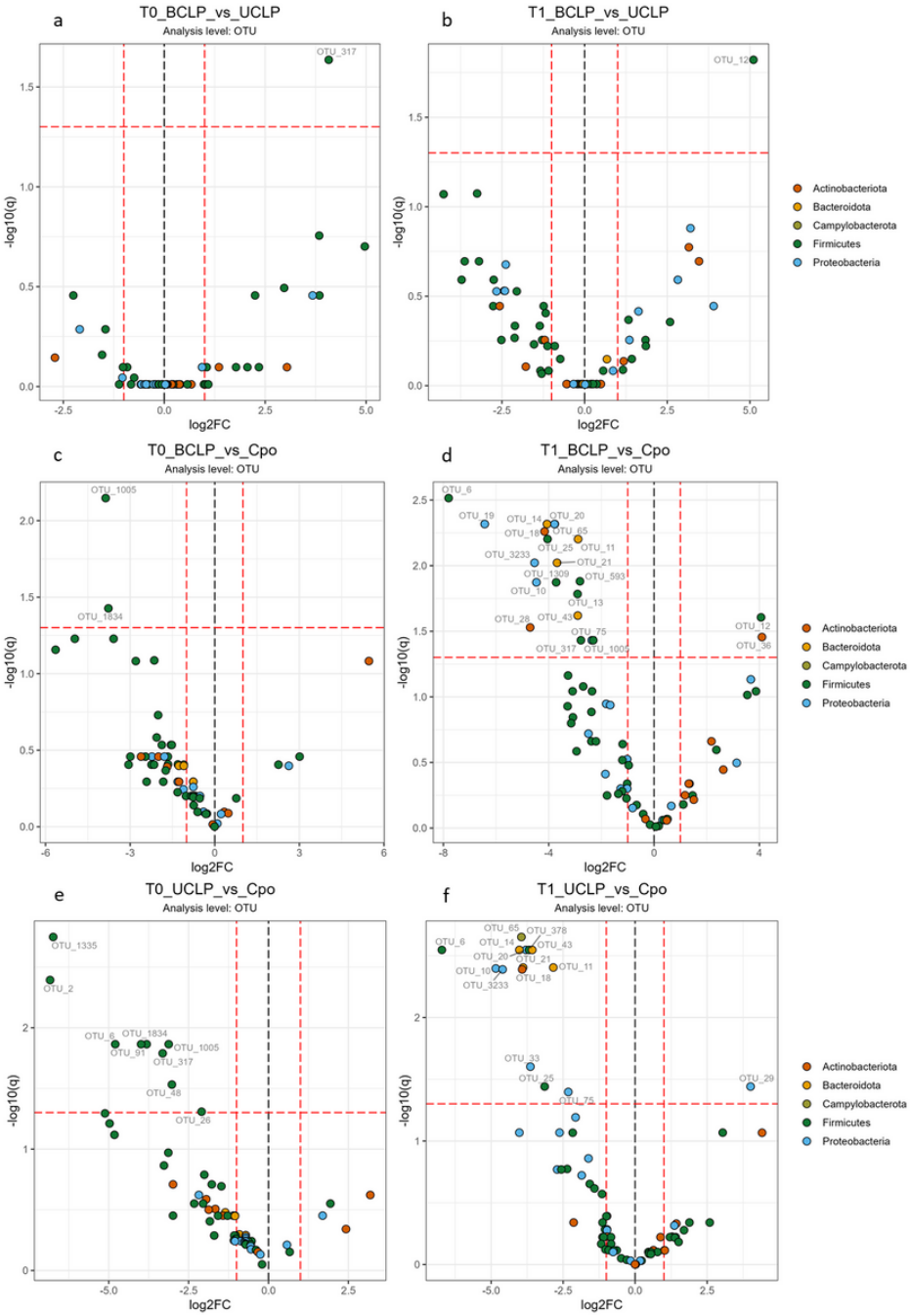

Figure 7

Differential analysis of Beta-diversity. Volcano plots on operational taxonomic (OTU) level is given for a) bilateral cleft lip palate (BCLP) vs. unilateral cleft lip palate (UCLP) at T0; b) BCLP vs. UCLP at T1; c) BCLP vs. CPo at T0; d) BCLP vs. CPo at T1; e) UCLP vs. CPo at T0; f) UCLP vs. CPo group at T1. Genus and OTUs are given next to colored dots, which follow a color scheme to visualize the corresponding phyla (orange dots = Actinobacteriota; yellow dots = Bacteroidota; light green dots = Campylobacterota; dark green dots = Firmicutes; blue dots = Proteobacteria) and dashed lines indicate significant differences (>2-fold, adj. p (q) < 0.05) between the comparators.

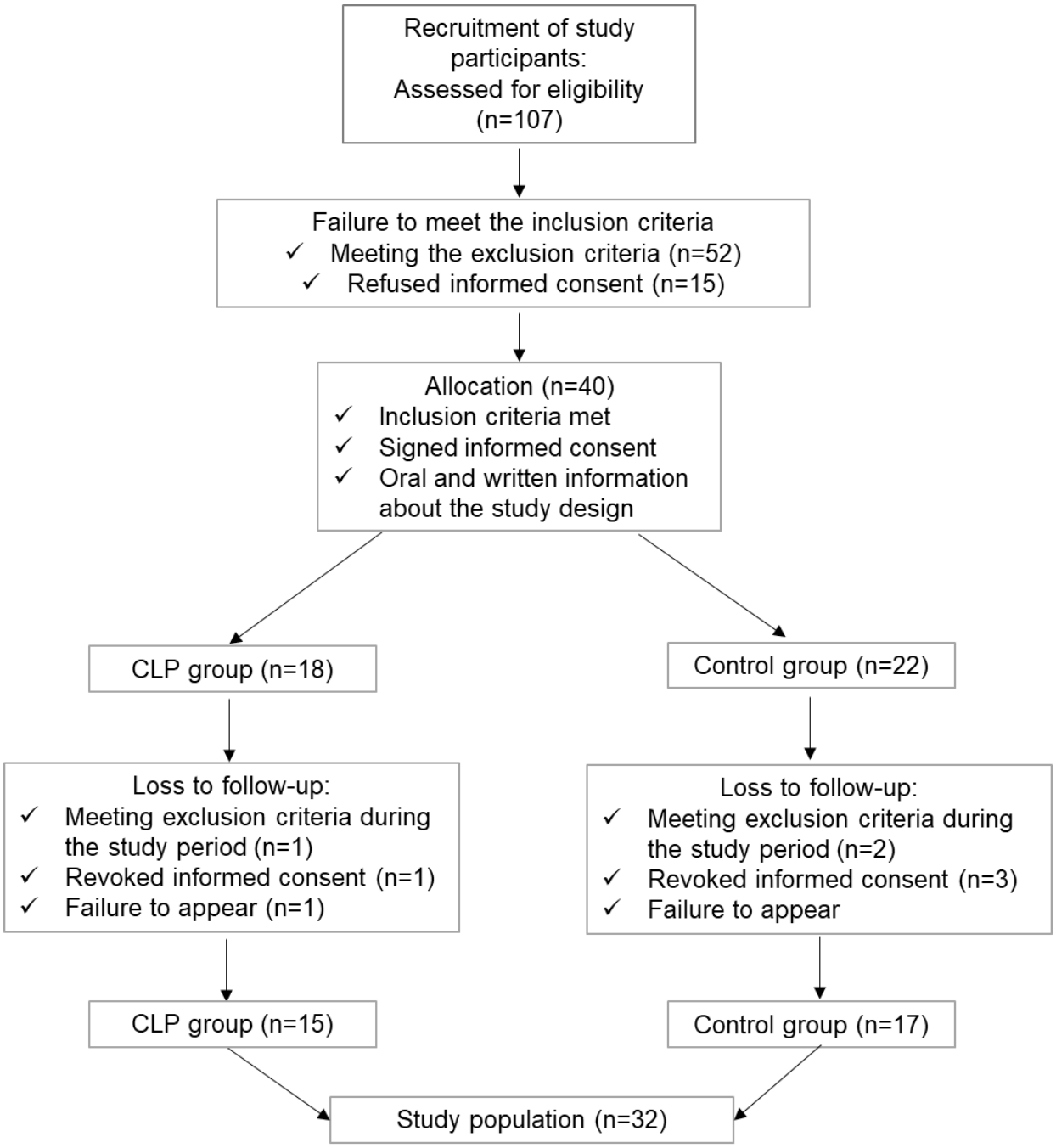

Figure 8

Flow of study participants