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by

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THE ORAL MICROBIOME IN THE SCOPE OF EARLY LIFE ADVERSITY

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Affidavit

I hereby confirm that the PhD thesis entitled "***The Oral Microbiome in the scope of Early Life Adversity***" has been written independently and without any other sources than cited. All necessary ethical approvals in regards to The EpiPath human cohort obtained by the Luxembourg National Research Ethics Committee (CNER, No 201303/10 v1.4) as well as the University of Luxembourg Ethics Review Panel (ERP, No 13-002).

Greifswald, 16.03.2023 Eleftheria Charalambous

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Dedication

In loving memory of my Grandfather.

My forever beacon of light.

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"No one who achieves success does so without acknowledging the help of others. The wise and confident acknowledge this help with gratitude."

- Alfred North Whitehead -

I would like to thank first the person who made this project possible, the person who believed in me, sometimes even more than my own self, the person who trusted me and gave me the possibility to embark on this journey. The person who every step of the way made sure to challenge my limits and made sure I was as comfortable as possible while marching the development zone. This is none other than my supervisor Dr. Jonathan Turner. Immediately after I would like to express my gratitude to the ALL6 team for all your support. Sophie and Pauline thank you for all the lab support. Nathalie, for being there when needed and all the nice ideas we exchanged for the animal protocol that never happened. Thank you to my office ladies, previous Sara, Myriam, Sneha, current Cyrielle and latest addition Archie, for making the office hours fun, enjoyable and for all your patience during my coding sessions. Also, Cyrielle in particular thank you for being the best coding, diving and conference buddy.

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Last, thank you to all those who encouraged me to spread my wings and fly in the direction of my dreams. Sometimes acknowledgment received when least expected is the stronger confidence boost.

"With a bit of faith and hard-work you can make miracles"

Eleftheria Charalambous, 12/12/2022

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List of Abbreviations

ACTH	Adrenocorticotropic Hormone
Ahcys	S-Adenosyl-L-homocysteine
Amet	S-Adenosyl-L-methionine
ATP	Adenosine Triphosphate
AU	Area Under the Curve
BH	Benjamini-Hochberg
BMI	Body Mass Index
CD	Cluster of Differentiation
CMV	Cytomegalovirus
COBRA	Constraint-based reconstruction and analysis
CRF	Corticotropin-releasing factor
DAG	Directed Acyclic Graph
DNA	Deoxyribonucleic acid
DOHaD	Developmental Origins of Health and Disease
EBV	Ebstein Barr Virus
EDTA	Ethylenediaminetetraacetic acid
ELA	Early Life Adversity
ELI	Early Life Infection
ELM	Early Life Microbiome
FNR	Fonds National de la Reserche
GLM	Generalised Linear Model
GPCR	G-protein-coupled receptor
HMOs	Human Milk Oligosaccharides
HPA	hypothalamus-pituitary-adrenal
HSPC	Hematopoietic Stem and Progenitor Cells
HSV	Herpex Simplex Virus
IBD	Inflammatory bowel disease
Ig	Immunoglobulin
IL	Interleukin

INF-I	Interferon type I
LPS	Lipopolysaccharides
NK cell	Natural Killer cell
NMN	Nicotinamide Mononucleotide
OM	Oral Microbiome
PND	Post-natal day
PWM	Pokeweed mitogen
QS	Quorum Sensing
RNA	Ribonucleic acid
SCFAs	Short Chain Fatty Acids
SECPT	Socially Evaluated Cold-Pressor Test
SEM	Standard Error of Mean
SES	Socio-economic status
SFB	Segmented Filamentous Bacteria
SLE	Systemic lupus erythematosus
STAT1	Signal Transducer And Activator Of Transcription 1
T1D	Type 1 diabetes
T2D	Type 2 diabetes
Th	T helper
TLR	Toll-Like Receptor
Tma	Trimethylamine
Tmao	Trimethylamine N-oxide
TSST	Trier Social Stress Test

Summary

In a way, the microbiome has been on earth long before we humans appeared in any evolution theory. This ethereal “organ” has grabbed the attention of biological research and will continue to expand in the years to come. Growing evidence supports that its presence is vital to the host and particularly to us humans. Although largest part of the mirobiome research focuses on the gut microbiome, the microbial communities actually stretch in the farthest off body sites. The last few years, oral microbiome (OM) has started to steal some light. Despite of the geolocation, the microbiome maintains an intimate relationship with its host. Our long term health appears to be closely linked and sustained by a “happy”, healthy and symbiotic microbiome.

Disease and state of illness, whether it is of physiological or psychological nature or both can result from many different causes and routes. Remarkably, about a third of the adult population worldwide seems to become ill as concequence of certain difficult, stressful and intense experiences during their childhood. The field of social, biological, medical and environmental sciences describes these experiences as “Early Life Adversity (ELA)” and the marrow of the “Developmental Origins of Health and Disease (DOHaD)” . Since the 80s that this sociobiological theory was introduced and described, research revealed that ELA factors could be anything from pregnancy complications, medication, birth complications, infections, social isolation, socioeconomic status, orphanhood, toxic environment and any other event that is capable of inducing extreme stress to an individual. Concequences of ELA experiences have also been introduced as wide range of common later life diseases.

An institutionalisation cohort as a model of ELA, the EPIPATH, which was initially established to study the longterm immune and cardiometabolic effect of institutionalisation was used for this Thesis. At first, I aimed to investigate what was the effect of institutionalisation on the OM of these individuals using their oral taxonomic composition. Secondly, I aimed to identify a link between this composition and the immune profiles of the participants. Thirdly, knowing the stress signatures of the cohort following pre-existing cortisol and glucose measurements, I aimed to detect plausible interaction between the microbiome and those data. Lastly, I aimed to find mechanistic evidence of our prior observed associations by looking into the metabolome of the oral microbiome.

Altogether our data revealed observations of a complex system of bidirectional interactions between ELA, the OM and the immune markers of cytotoxicity and immune

senescence together with a particular profile of glucose and cortisol kinetics following exposure to social stress. Besides, our data expanded to the exposure of the first mechanistic cues of ELA traces on the OM metabolome. The research conducted for this thesis brings to light important evidence on how ELA interacts with the OM leading to a certain disease phenotype.

Chapter 1. Introduction – Identifying the jigsaw pieces

Parts of this section have been adapted from the following manuscripts:

Charalambous EG, Mériaux SB, Guebels P, Muller CP, Leenen FAD, Elwenspoek MMC, Thiele I, Hertel J & Turner JD. Early-Life Adversity Leaves Its Imprint on the Oral Microbiome for More Than 20 Years and Is Associated with Long-Term Immune Changes. Int J Mol Sci. 2021 Nov 24;22(23):12682. doi: 10.3390/ijms222312682. PMID: 34884490; PMCID: PMC8657988.

Holuka C, Merz MP, Fernandes SB, **Charalambous EG**, Seal SV, Grova N & Turner JD. The COVID-19 Pandemic: Does Our Early Life Environment, Life Trajectory and Socioeconomic Status Determine Disease Susceptibility and Severity? Int J Mol Sci. 2020 Jul 19;21(14):5094. doi: 10.3390/ijms21145094. PMID: 32707661; PMCID: PMC7404093.

1.1. Early life Adversity

Early life experience of multifactorial adverse factors, including toxic and poor socio-economic environment, orphan-hood, malnutrition and infections result to a psychophysiological and emotional strain that drives adult-onset disease (Grova et al., 2019, Turner, 2018a, Turner, 2018b, Zhong et al., 2022). Barker and Osmond in the mid-1980s were the first to introduce the developmental origins of health and disease (DOHaD). In this concept, the environment in the first 1000 days was hypothesised to be a driver of health and disease profiles lifelong (Barker and Osmond, 1986). These first 1000 days cover from conception to 2 years which is considered the most vulnerable life period (Barker and Osmond, 1986). At birth, the body is almost fully formed; however, many biological systems continue to mature over the following years. Research on the lifelong health and disease

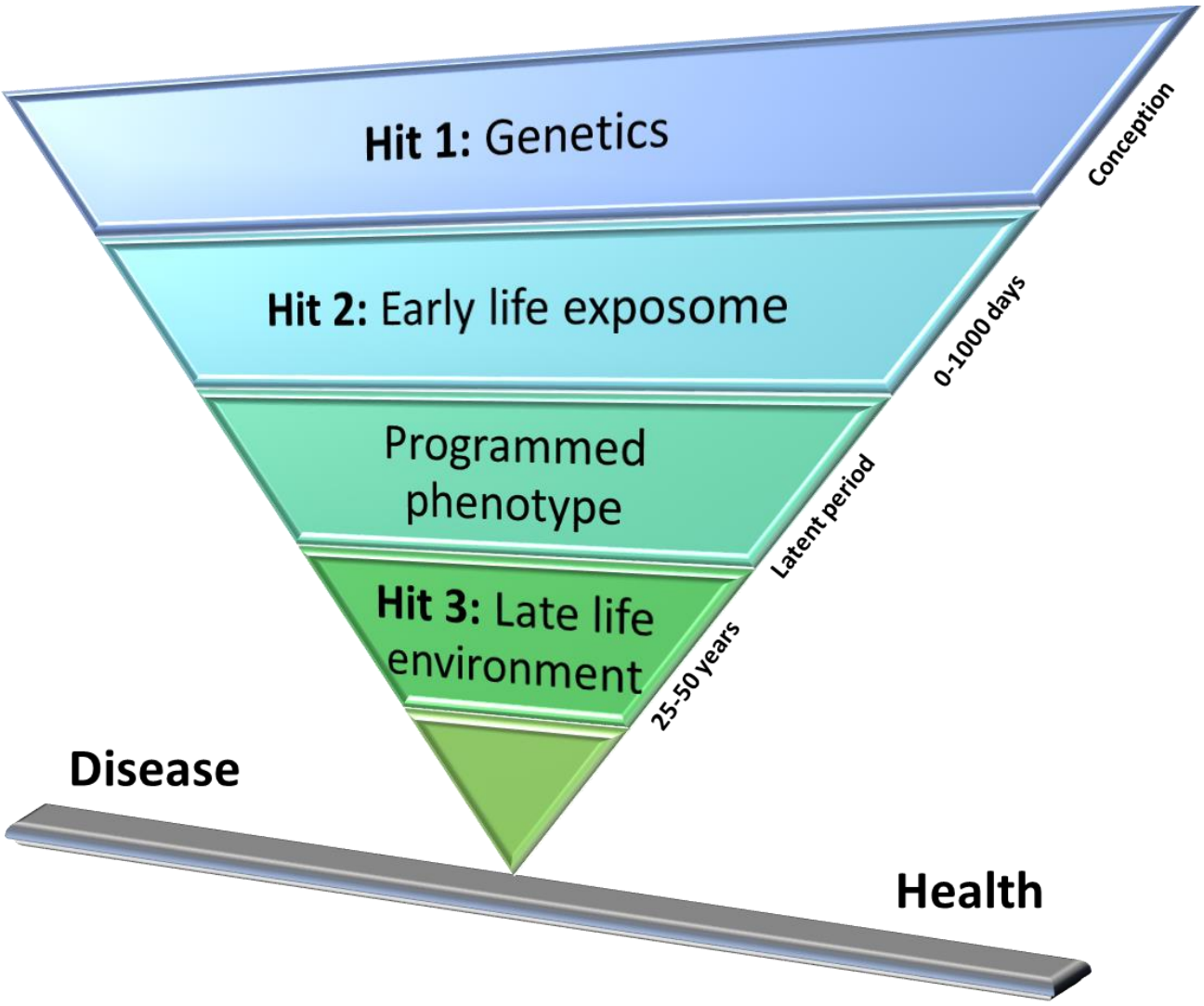

Figure 1.1. Three-hit model adapted from Daskalakis et al., 2013 (Daskalakis et al., 2013).

balance has shown the significance of the environment during this period on multiple disease phenotypes (Dube et al., 2009), including cardiovascular, allergic, and autoimmune disorders, as well as mental disorders (Reyman et al., 2019, Elwenspoek et al., 2017b, Wampach et al., 2018, Mansuri et al., 2020, Herzog and Schmahl, 2018, Gern et al., 2009, Tomasdottir et al., 2015, Eriksson et al., 2014, Backhed et al., 2015, Moore and Townsend, 2019, Yang et al., 2016, Abel et al., 2018). In 2013, Daskalakis et al., described the ELA to follow a 3-hit model where 1st hit depends on the genetics starting from conception, 2nd hit depends on the early life exposome of the first 1000 days and 3rd hit depends on the trigger in later adulthood that results in the ELA phenotype (Daskalakis et al., 2013).

Primary research focus of ELA since the 1980s has been the molecular mechanisms and the cellular phenotype behind the effect of stress and adversity on immune and endocrine systems as well as epigenetic modifications (Reyman et al., 2019, Elwenspoek et al., 2017b). Multiple reports suggest that ELA influences health trajectories via the immune system (Elwenspoek et al., 2017b, Holland et al., 2020, Reid et al., 2019), with a clear ELA-associated immunophenotype centred around the activation and functional status of T lymphocytes. In the institutionalisation model of early-life stress, strong T-cell immunosenescence has been reported (Elwenspoek et al., 2017b, Reid et al., 2019, Elwenspoek et al., 2017c). Immunosenescence is a form of accelerated immune ageing. The CD57 T- and NK- cell immunosenescence marker is absent in early life and increases with age, with high numbers of such cells in the elderly population. Immunosenescence is driven by chronic inflammation or recurrent viral infections such as CMV (Nielsen et al., 2013). NK functionality is also highly impacted by recurrent reactivation of CMV inducing NK cell exhaustion, increased cytotoxicity, and senescence (Judge et al., 2020). Additionally, such viral infections potentially program the immune system (Reid et al., 2019, Elwenspoek et al., 2017c). Latent CMV infection of haematopoietic progenitor cells reduces GR transcription and translation, impacting immune cell maturation, which can be dependent on CMV reactivation (Elwenspoek et al., 2020, Della Chiesa et al., 2012, Lopez-Verges et al., 2011).

In later years, research interests shifted towards the stress phenotype accompanying ELA- associated diseases like type 2 diabetes (T2B). ELA is intimately linked to stress. In the exposure to a stressor, the autonomic nervous system and the HPA axis activate and release of catecholamines and glucocorticoids which are the coordinates of the “fight or

flight" response (Seal and Turner, 2021, Seal et al., 2022, Bowland and Weyrich, 2022). At the same time, glucose is produced and released from the liver (Seal and Turner, 2021). Exposure to ELA interferes lifelong with with HPA axis regulation and glucocorticoid levels and consequently affects glucose release and metabolism dynamics (Seal et al., 2022, Hengesch et al., 2018). It is speculated that this process is mediated by glucocorticoid signalling. Yet, how this process is altered has not been well studied.

In summary, the intense stress induced by ELA leads to a multisystem crosstalk so far including immune system functioning and HPA axis regulation with neuroendocrine and metabolic changes.

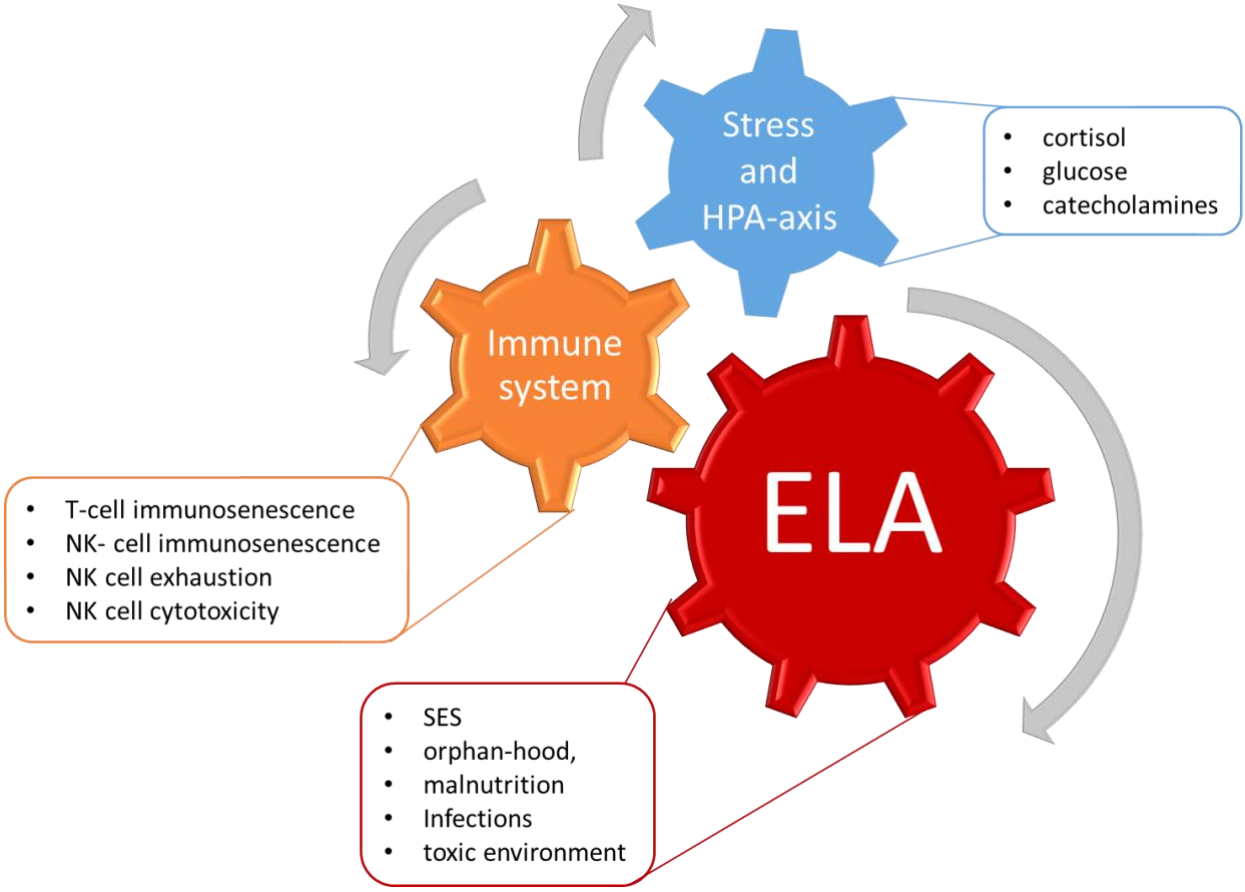

Figure 1.2. ELA – Immune system – HPA axis schematic interaction.

Nowadays, at least a third of the population globally has experienced ELA between 0-7 years old. Due to the sensitivity of ELA experiences, many go undocumented and globally statistics vary between countries and geographic regions. Although ELA occurrence is much more frequent in developing countries, prevalence in developed

countries is increased. For instance, as identified by Carlson et al in 2019 from reviewing 52 US studies from a period of 15 years and including social adversities reported in young children and teenagers, prevalence extended from 41% to 97% (Boney-McCoy and Finkelhor, 1995, Kilpatrick et al., 2000, Carlson et al., 2020). Whilst occurrence of trauma events varies between types and in addition to psychosocial adversity include ecological, biological, physical, lifestyle and other epidemiological types (Merz and Turner, 2021). The long lasting impact of ELA can be seen as early as adolescence to middle adulthood. Kessler et al in 2010, claimed that 29.8% of health disorders observed between 21 countries were consequences of childhood adversity (Kessler et al., 2010). Several studies have already tracked the roots of long lasting psychophysiological conditions to ELA (Wampach et al., 2018, Ling et al., 2020, Reyman et al., 2019, Sarkar et al., 2021, Moore and Townsend, 2019, Backhed et al., 2015, Eriksson et al., 2014, Spitzer et al., 2013, Tomasdottir et al., 2015, Gern et al., 2009, Herzog and Schmahl, 2018, Mansuri et al., 2020, Rod et al., 2021)

One of the most recent form of ELA experienced globally is Covid19 pandemic which covers all types of adversity. Infection with covid19 at the first 36 months of age had a tremendous stress on the developing immune system of the babies (Gotzsche et al., 1988). Maternal infection with covid19 prior to birth consists of intense pre-natal stress with diverse postnatal outcomes while post-natal isolation essential to limit viral spread impacted horrifically maternal care and nurturing parental habits (Xiong et al., 2023). Investigations from 20 months into the pandemic are now showing that these babies born during the covid19 outbreak show a diminished physical and verbal as well as overall cognitive performance in contrast to pre-pandemic born infants (Deoni et al., 2022, Xiong et al., 2023). In addition, the drastic hygiene measures that were applied worldwide forced infants into a long social isolation, extensive maternal distress result to a disrupted socioemotional development with an undefined period (Xiong et al., 2023, Davies et al., 2021, Duguay et al., 2022). To a large extend, the pandemic experience has taken away everything that consists a “healthy environment”. Not only introduced additional stress and uncertainty in the society, it also emphasised and strengthen socioeconomic inequalities affecting primary medical, nutritional and educational needs (Gotzsche et al., 1988, Bang, 2023, Jaramillo and Felix, 2023, Ogundipe et al., 2023).

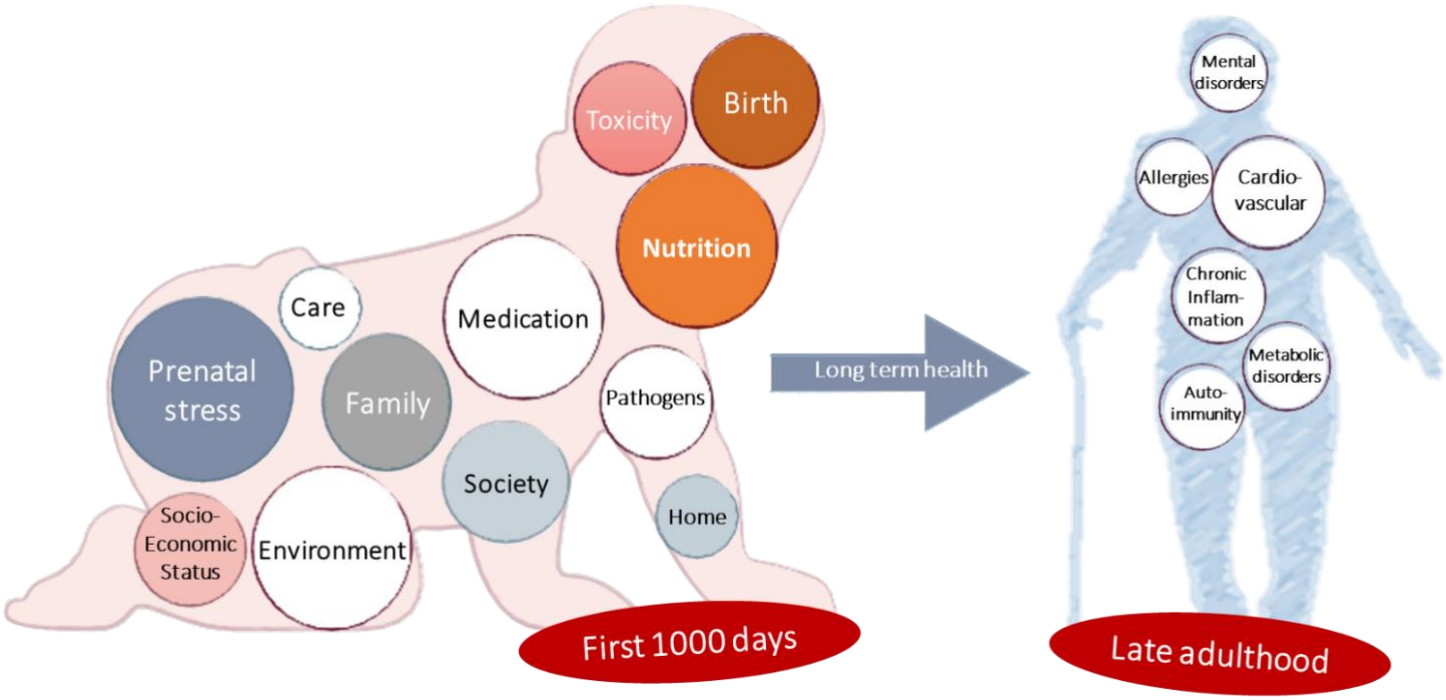

Figure 1.3. The meaning of ELA in DOHaD.

Altogether, ELA experiences are widely present and help drive human heterogeneity. For instance, they are the trigger of health state tipping onto disease state in the long term. They are the aetiology of diversity in disease symptomatology and disease progression and management. The reason behind, is likely to be trauma dependent as each experience and intensity of experience carve a certain molecular mechanism or a network of molecular mechanisms with diverse developmental and psychopathophysiological concequences. Studies on the impact of adversity to the DOHaD are linking biological and social sciences, public health and epidemiology as well as socioeconomic and human rights experts. While ELA has been established as a causal link to many long lasting psychophysiological conditions (Wampach et al., 2018, Ling et al., 2020, Reyman et al., 2019, Sarkar et al., 2021, Moore and Townsend, 2019, Backhed et al., 2015, Eriksson et al., 2014, Spitzer et al., 2013, Tomasdottir et al., 2015, Gern et al., 2009, Herzog and Schmahl, 2018, Mansuri et al., 2020, Rod et al., 2021, Turner, 2018b, Miller et al., 2018, Nigg et al., 2020), twin studies are emphasizing on how the adversity can influence the long-term health of genetically identical individuals (Castillo-Fernandez et al., 2014, Bowyer et al., 2019, Miller et al., 2016, Azad et al., 2013, Yatsunenko et al., 2012, Czyz et al., 2012, Vaiserman, 2015, Talens et al., 2012,

Turner, 2018b, Nigg et al., 2020). More recently ELA research has expanded to include the whole experience of adversity which can vary in twins, leading to a divergent psychopathophysiological and ecological profile in their later life (Castillo-Fernandez et al., 2014, Bowyer et al., 2019, Miller et al., 2016, Yatsunenko et al., 2012, Czyz et al., 2012, Talens et al., 2012, Turner, 2018b, Nigg et al., 2020, Galetzka et al., 2012).

Inevitably all the above leads us to the conclusion that the foundation of the ELA-OM interactions start as early as the time of first tissue establishments. During this very labile period many site specific tissues continue to further develop along with programming of tissue specific resident cells as well as seeding and development of the early life microbiome. Following ELA, an altered cellular imprint and epigenetic profile has been seen in adipocytes alongside with inflammatory markers, altered brain structure and impacted connectivity of brain regions like the amygdala, prefrontal cortex, frontal and parietal, anterior cingulate cortex, striatal, insula, hippocambal, precuneus, and limbic together with methylation patterns have been reported from multiple studies (Chu et al., 2018, Coley et al., 2021, Kerns et al., 2004, Menon and Uddin, 2010, Xu et al., 2019). Furthermore, patterns on limphoid tissue and differentiation of immune cells together with site specific mucosal barriers have also been reported (Campbell et al., 2023). The immune system is somehow involved in majority of reports and probably the core mechanism may be related to immune tolerance which occurs during this period leading to the maturation of immune limphoid tissues (Campbell et al., 2023). Simultaneously the newly residing and developing microbiome is now recognized as “commensal” in order to prevent immune responses against it and supports the host to only distinguish and specialise against invasive pathogens (Campbell et al., 2023, Wang et al., 2019, Minarrieta et al., 2017, Levy et al., 2017).

Evidently, the ELM including the OM have a pivotal role on the development of immune tolerance and maturation of the immune system. Additionally, the presense of the ELM in the various body sites provides the host with microbial metabolites which are equally valuable for host`s metabolism and HSPCs differentiation and myeloid cell priming via signaling to the bone marrow (Dang and Marsland, 2019, Yan et al., 2022, Trompette et al., 2014, Trompette et al., 2018). Such evidence mostly exist from studies on gut-lung microbiome and mucosal barriers. Investigations on the impact of OM on the early life devepment are yet to be conducted and bring to light additional evidence.

1.2 Oral microbiome

A few hours after birth, the first body-area-specific microbial communities are seeded and start to develop. Once established, the microbiome modulates the host (Yang et al., 2009), a mechanism to protect symbiotic microbial communities, where cases of microbial dysbiosis can be fatal (Wang et al., 2020, Rogers et al., 2016). The ELM plays important roles in an infant's subsequent development (Wampach et al., 2018, Shao et al., 2019) and a long-term health trajectory (Wampach et al., 2018, Shao et al., 2019, Reyman et al., 2019, Sarkar et al., 2021, Yang et al., 2016, Moore and Townsend, 2019, Backhed et al., 2015). Nevertheless, the development of the ELM is critically impacted by ELA factors and the environment including mode of birth, early-life nutrition, and environmental exposure, which leave a clear lifelong trace (Herzog and Schmahl, 2018, Miller et al., 2018). For example, maternal milk is rich in essential nutrients, protective antibodies, and components essential for the developing microbiome, such as human milk oligosaccharides (HMOs) and short-chain fatty acids (SCFAs) (Zijlmans et al., 2015, Alcon-Giner et al., 2020, Stewart et al., 2018, Xu et al., 2020, Toussaint et al., 2016, Duran-Pinedo et al., 2018).

Over the last decade it has become clear that once the microbiome is established it is shaped by the exposome and the ~9 million microbial genes it encodes and play a crucial role in determining host development and health (Tomasdottir et al., 2015, Elwenspoek et al., 2017a, Fernandes et al., 2021, Reid et al., 2021). Modulating the host most probably protects the natural enteric symbiotic microbial community, and disturbing the established microbiome, producing a dysbiosis, results in disease and may even be occurs during this *"DoHAD"* sensitive period in which the new-born immune system is primed (Gomez et al., 2017), may explain why babies born by caesarean section have a significantly increased risk of allergy or asthma later in life (Premaraj et al., 2020b). Exposure of new-borns to a more diverse microbiota soon after birth altered both the disease susceptibility and maturation of specific immune cell subsets, whereas if the first encounter occurred later, immune dysfunction was not corrected (Blekhman et al., 2015b, Byrd and Gulati, 2021). Regulatory T cells (T_{reg}) play a significant role in the host adaptation to the microbiome, recognize host-specific commensal bacteria derived antigens (Gomez and Nelson, 2017), and result in long-

term tolerance to the enteric microbiome (Li et al., 2000). It would appear that adverse microbiota is essential for the immune system to fully mature (Li et al., 2016).

With the rise in the importance of the microbiome, now ELA mechanistic research is shifting its focus towards the microbiome. Emerging evidence report that dysbiosis on maternal microbiota, ceasarian section microbiome, exposure to infection and necessary use of antibiotics either at prenatal period or soon after birth are associated with pregnancy complications and later life illness (Yao et al., 2021, Patangia et al., 2022). Implying that, in a way the microbiome is impacted by ELA and it is also a factor belonging to ELA context.

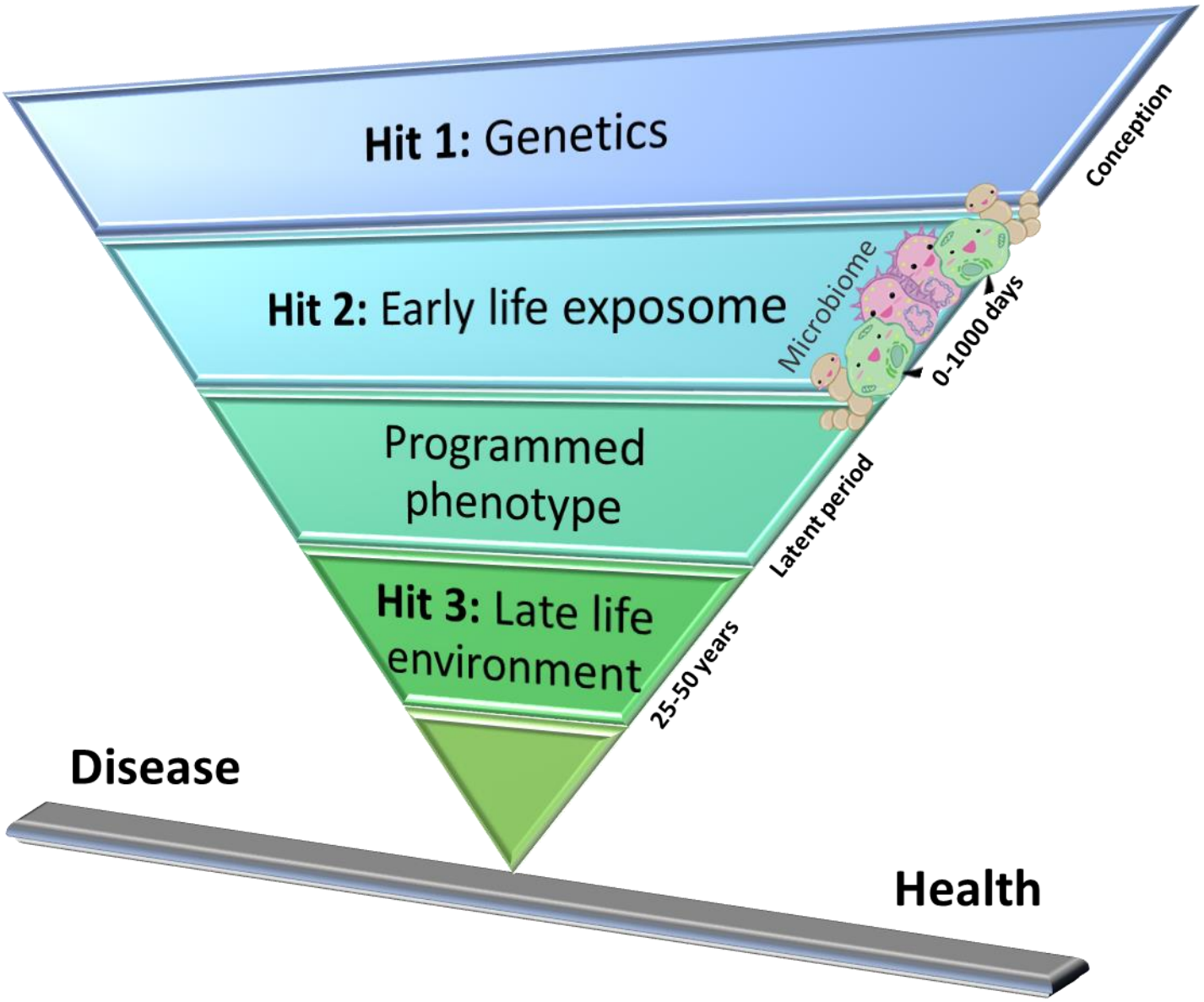

Figure 1.4. The Oral Microbiome on the 3-hit model adapted from Daskalakis et al., 2013.

The oral microbiome (OM) is composed of various distinct, smaller communities within the oral cavity (Boustedt et al., 2015, Mark Welch et al., 2019, Carpenter, 2020) that are robust, stable, and particularly resilient (David et al., 2014, Shaw et al., 2017), particularly to antibiotic therapy (Shaw et al., 2017, Marsh, 2006, Zaura et al., 2015, Kennedy et al., 2019, Abeles et al., 2016). Moreover, saliva contains actively secreted components such as cortisol, glucose, lactate, urea, and proteins, such as polypeptides, glycoproteins (cystatins, mucins, and immunoglobulins) and antimicrobial peptides (histatins, defensins, and immunoglobulins-IgA). Many of these are energy sources for the OM, and salivary glycoproteins are the principal nutrient source. These substrates are crucial for the development of multispecies communities and their preservation (Mark Welch et al., 2019, Kennedy et al., 2019, Almeida-Santos et al., 2021), and enhance the resistance of the community to environmental stressors (Carpenter, 2020, Mukherjee et al., 2021, Jakubovics, 2015). The long-term stability of the OM leads to the hypothesis that, once established in early life, it remains stable, robust, and resilient, retaining an imprint of the early environment (David et al., 2014, Shaw et al., 2017). According to the 3-hit ELA model described previously, the OM belongs in the "second-hit" and continues to develop over the first 36 months (Daskalakis et al., 2013, Socransky and Manganiello, 1971) .

Within this context, recent studies are looking for long-term changes in the microbiome. In the institutionalisation-adoption model of ELA there is now a clear link between increased senescence in T-cells that is associated with a specific taxonomic profile of the microbiome (Elwenspoek et al., 2017c, Holland et al., 2020, Reid et al., 2019, Charalambous et al., 2021). In addition, there have been reports from the same model of a microbiome-immune crosstalk where microbiome members associate with the senescent CD57 marker and other immune cell activation markers (Reid et al., 2021, Charalambous et al., 2021). Other studies on early life development showed that the early oral microbial colonisation associates to IL-17-producing cells (Koren et al., 2021) while investigations on host-microbe interactions in relation to chronic oral disease showed the microbiome to drive a Th17 cells and IL-17 shifted immune response (Bellando-Randone et al., 2021, Abusleme and Moutsopoulos, 2017, Gaffen and Moutsopoulos, 2020).

Evidently, the OM is intimately linked to oral health. Poor oral health is often approached in an eco-social framework, as it is known to be associated with psychosocial adversity (Lee and Divaris, 2014). Both epigenetic and behavioural pathways were linked

to poor oral health (Lee and Divaris, 2014). One of the most studied causal routes is diet. Affordability and access to a nourishing diet are strongly influenced by socio-economic status (Lee and Divaris, 2014), which in turn is linked to the composition of the OM. Detrimental shifts in the microbial composition associated with poor immune responses and mental health were documented for both hospitalised and long-term care home residents (Coman and Vodnar, 2020). The multidirectional interconnected relationship between the microbial composition, the host's immunological status, and the resulting life-long health trajectory is most probably highly dependent on constant exposure to particular irritants (Coman and Vodnar, 2020).

In fact, the evidence of a microbiome-immune crosstalk have always been there. In 1971, Socransky and Manganiello were the first to characterize the OM (Socransky and Manganiello, 1971). Since then there have been multiple reports regarding the appearance of oral diseases and signs of oral inflammation after the teething was complete from children to adults (Lee and Divaris, 2014). Neither the microbial profile nor the mechanisms behind this interaction have been in the centre of attention, until recently.

1.3. Microbial metabolites

The OM like all bacterial communities has the capacity to metabolise nutrients producing the microbial metabolites. These are chemical and/or carbon-based molecular compounds derived from energy and survival focused microbial metabolism (Takahashi, 2015, Krautkramer et al., 2021, Horak et al., 2019). The OM "metabolome" is defined by the metabolite pool of each individual microbe or the metabolite pool of the particular microbial community (Horak et al., 2019, Tang, 2011, Aldridge and Rhee, 2014). This is further categorised into primary and secondary metabolites and consisted by different classes of composites including carbohydrates, sugars, proteins, peptides, amino acids, short-chain fatty acids and other acids or alkaline products which are often recycled within metabolic pathways (Horak et al., 2019, Pinu et al., 2017, Villas-Bôas, 2007). These metabolites are equally important to the individual microbes, to the whole microbiome community and to the host (Krautkramer et al., 2021, Horak et al., 2019). Microbial metabolites complement the host`s endogenous metabolism, and growing evidence suggests that are capable of modulating host physiology. Besides, bacterial metabolites can induce epigenetic modifications, which subsequently can either activate or inhibit the immune

responses and interfere with the host`s neuroendocrine system, impact the intestinal mucosal surface, and also can pass through the blood-brain-barrier (Connell et al., 2022, Bowland and Weyrich, 2022, Narengaowa et al., 2021, Dong et al., 2022).

The capacity of the OM to generate microbial metabolites appears to be the connection of this microbiome-immune crosstalk. Before achieving the crosstalk though, with commencement of primary nutrients digestion in the oral cavity, fermentable carbohydrates are metabolised by oral bacteria and produce short chain fatty acids (SCFAs) (Barbour et al., 2022, Campbell et al., 2023, Parada Venegas et al., 2019). In addition, OM utilises other available molecules originating from salivary mucins via various carbon associated pathways in order to produce pyruvate, important for downstream ATP generation. Pyruvate is also further metabolised via various pathways into SCFAs (Barbour et al., 2022, Takahashi, 2015). OM is also capable of producing SCFAs from fermentation of aminoacids which are dominant in various locations of the oral cavity (Barbour et al., 2022, Norimatsu et al., 2015, Tsuchida et al., 2018). SCFAs production is so important for the OM due to their antimicrobial potential that helps to maintain symbiosis between the OM community members and also provide protection for both the OM and the host against pathogens (Barbour et al., 2022, Campbell et al., 2023, Georgios et al., 2015, de Sablet et al., 2009). The role of SCFAs and other metabolites produced from the OM is far greater and extends much further beyond the homeostasis of the oral cavity. Of course, homeostasis within the oral cavity is of primary importance as ensures a happy symbiotic microenvironment between OM members and their host.

To ensure local and systemic homeostasis, the key players behind the microbiome-immune crosstalk are these microbial metabolites. Locally, evidence suggest that these stimulate in a toll-like receptor (TLR) dependent mechanism the recruitment of innate cells, monocytes and neutrophils as well as promote expansion of the leukocyte recruitment using the metabolite hydrogen sulfide (H2S) (Barbour et al., 2022, Bitschar et al., 2019, Basic et al., 2019). Furthermore, in vitro studies of oral diseases suggest that hyper-inflammatory responses can potentially be control via SCFAs such as butyrate (Barbour et al., 2022, Magrin et al., 2020).

Whilst OM research is only starting to expand now and there is not definitive evidence on associated mechanisms, it is assumed that the OM behaves in a similar way as

other more widely studied communities like the gut microbiome. For instance, microbial metabolites in the gut are known to be capable of regulating the intenstinal permeability via different immunomodulatory mechanisms (Campbell et al., 2023, Mager et al., 2020). Although understudied, it is likely that OM can also modulate barriers of the oral mucosa. In addition, iosine from gut bacteria enhances the differentiation of naïve Th1 cells (Campbell et al., 2023, Mager et al., 2020). Likewise, certain SCFAs are able to affect both innate and adaptive immunity by driving the polarization of M2 macrophages and the suppression of Th17 cell differentiation with a systemic impact (Campbell et al., 2023, Sun et al., 2023). It is therefore highly plausible that OM metabolites are having very similar immunomodulatory properties.

To expand the systemic impact of the microbial metabolites, the SCFAs are believed to mediate microbial-host signalling while evidence associates them to regulation of glucose homeostasis and an improved insulin response (Vieira Lima et al., 2022, Wu et al., 2017a). On the contrary, in the absence of SCFAs, metabolites produced from fibre fermentation by the local microbiome communities, certain CD4 T-cell subsets do not differentiate. Furthermore, naïve CD8 T cells do not differentiate into memory cells in germ-free mice (Zheng et al., 2020, Smith et al., 2013, Bachem et al., 2019). One of the signalling pathways the SCFAs are known to use is GPCR signalling (Giuffre et al., 2020, van de Wouw et al., 2018, Kim, 2023). Moreover, histone modifications induced via SCFAs could be a double-edge sword while are found associated to disease pathogenesis and simultaneously may exhibit anti-tumorigenic and anti-inflammatory effects (Giuffre et al., 2020, Kim, 2023, Tan et al., 2014, Sharma and Taliyan, 2015, Dang and Marsland, 2019, Mizuno et al., 2017, Park et al., 2016, Campbell et al., 2023). Rising evidence proposes of an interaction between the microbiome and bone marrow through microbial metabolites (Dang and Marsland, 2019, Woo and Alenghat, 2022). Studies from animal models make evident this concept through data that show certain microbial metabolites to shape the immune profile via direct methylation-demethylation on bone marrow derived immune progenitors (Woo and Alenghat, 2022, Burgess et al., 2016, Burgess et al., 2020). Further mechanistic studies demonstrate that this regulation of hematopoiesis of the microbiome is dependent on INF-I Interferon type I and STAT1 signalling (Yan et al., 2022, Halupa et al., 2005, Demerdash et al., 2021).

And yet, if ELA can change the composition of the OM, it may also alter its metabolic programming. Therefore, we propose that in the context of ELA-OM interaction, the consequences are shaping of the immune system and tolerance, but also the microbial metabolome (Brandtzaeg, 2013). And so, attention falls on the microbiome and its metabolites. Research is now expanding to consider the OM and OM metabolome an environmental mediator and moderator of ELA associated diseases (Cox et al., 2022, Dube et al., 2009, Eriksson et al., 2014, Tomasdottir et al., 2015, Spitzer et al., 2013, Gern et al., 2009, Herzog and Schmahl, 2018, Mansuri et al., 2020, Wampach et al., 2018, Shao et al., 2019, Sarkar et al., 2021, Moore and Townsend, 2019). To summarize, in the context of ELA where we have both an immunophenotype and an associated behavioural phenotype the succeeding hypothesis is the presence of a complex intertwined network of molecular mechanisms affecting tissue specific biological programming. During the exposure to ELA,

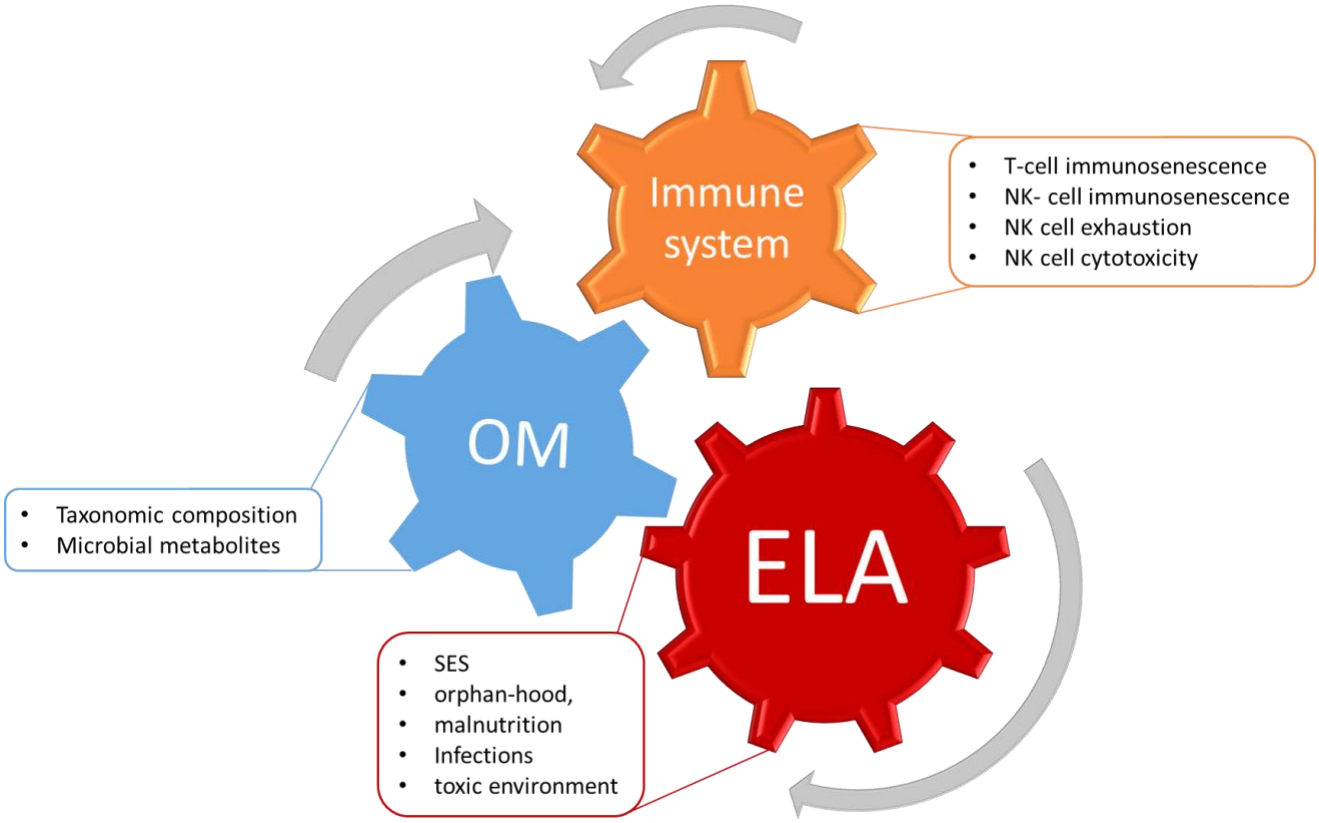

Figure 1.5. ELA – OM – Immune system schematic interaction.

the microbiome and microbial metabolome interacts with the global development of the immune system, nervous system and brain as well as metabolic programming.

1.4. OM in the systemic health and adversity concept

Interestingly, microbiome-immune interaction is observed already from the prenatal period. During fetal development, maternal microbiota provide microbial metabolites to the fetus that are utilised in multiple developmental stages including formation and development of fetal thymus (Hu et al., 2019). This may explain how pregnancy complications that are associated with maternal microbial dysbiosis may have adverse concequences for the fetal immune (Hu et al., 2019). This is a process that continues during the early neonatal period where microbial metabolites from breast milk and the newborn`s early life microbiome are utilised in postnatal immune development. This magnifies the importance of the maternal microbiota for the offspring`s postnatal development and long-term health (Campbell et al., 2023, Yoshida et al., 2018, Ahmadizar et al., 2018, Tapiainen et al., 2019). Key moderators of the microbiome-immune axis are the microbiome-HPA axis which initiates the immune signalling cascade and the microbiome-bone marrow axis where there is direct activation and differentiation of immune cell progenitors. The early life OM profile depends on maternal microbiota and the birth route, while its subsequent development is also highly dependent on early life nutritional intake (Lif Holgerson et al., 2011).

Originally, OM attracted the attention in order to bring light in the dental world to understand diseases observed in the oral cavity (Socransky, 1970). Much later it was found involved in psychosocial adversity as affordability and access to a nourishing diet" are strongly influenced by socio-economic status (Lee and Divaris, 2014), which in turn is linked to the composition of the OM. Observational studies linked the OM with cardiometabolic, mental, autoimmune and allergic diseases (Coman and Vodnar, 2020, King et al., 2022, Hajishengallis and Chavakis, 2021, Sedghi et al., 2021, Perez-Garcia et al., 2022). Mechanistic studies link epigenetic and behavioural pathways to poor oral health (Lee and Divaris, 2014). Interestingly many oral health associated systemic diseases have also been classified as ELA-associated (Reid et al., 2021, Elwenspoek et al., 2017b, Elwenspoek et al., 2017c).

Starting from a young age, studies report that children living in low SES have a higher risk developing dental caries and refuge children show an increase incidence of oral diseases together with ELA psychophenotype (Yousaf et al., 2022, Alhaffar et al., 2019, Hamid and Dashash, 2019). In addition, metagenomics studies identified an OM profile associating to the SES and oral diseases (Dashper et al., 2019). Disease oriented studies, in particular in type 1 diabetes (T1D children) disclosed a T1D oral microbiome profile which changes depending on the severity of T1D (Yuan et al., 2022). Adult-focused studies validate such reports and expand on broad range of ELA systemic diseases including: T2D, obesity, psychological diseases like emotional stress, depression and nicotine dependence, autoimmune diseases such as systemic lupus erythematosus (SLE) extending to arthritis, osteoporosis, late-life Alzheimer`s and Parkinson`s diseases and even gut-linked inflammatory bowel disease (IBD) as well as colitis (Li et al., 2000, de Lima et al., 2020, Albandar et al., 2018, Hajishengallis and Chavakis, 2021, Chelakkot et al., 2018, Sedghi et al., 2021).

The exact direction of oral-health to systemic-health OM interaction has not been well defined and it remains unclear what comes first as either can worsen the other`s progression and severity. For instance diabetes studies with OM profiling suggest that poor oral health can lead to diabetes whilst uncontrolled hyperglycaemia in diabetic patients contributes to periodontitis severity and bone loss, suggesting that the connecting mechanism lays in glycaemic control (Qin et al., 2022, Vieira Lima et al., 2022). Oral microbial metabolites they appear to be key mediators in the maintenance of oral and systemic health (Fraser and Ganesan, 2023). Lessons from the gut microbiome propose that ELA leads to an ELA taxonomic composition which is further manipulated by diet, external pathogens and medication. Subsequently this ELA microbial profile can cause epithelial damage resulting to the release of toxic microbial metabolites. Consequently, local and systemic inflammation is triggered resulting to disease (Chelakkot et al., 2018). Additional evidence support that microbial metabolites like bile acids, SCFAs, histamine, kynurenines, melatonin, and neurotransmitters can cross and travel through to the brain, via BBB crossing (Connell et al., 2022, Ling et al., 2020, Liu et al., 2020, Saji et al., 2019, Vogt et al., 2018, Li et al., 2018). For instance, trimethylamine N-oxide (TMAO), when found in increased levels in the brain is associated with cognitive decline. Remarkably, TMAO is produced in two-steps: first its progenitor TMA starts from the gut, then migrates to the liver where is

converted into TMAO and then it travels to the brain (Connell et al., 2022). Normal levels of TMAO show neuroprotective effect and elaborate in hormonal homeostasis, cholesterol metabolism and platelet hyperactivity (Connell et al., 2022).

Microbiome research so far proposes the existence of interactions on gut-brain axis, a gut-liver axis, a gut-lung axis, gut-bone marrow, oral-gut-liver axis, oral-gut-brain axis and oral-brain axis (Connell et al., 2022, Bajaj, 2019, Liu et al., 2021, Dang and Marsland, 2019, Hung and Matute-Bello, 2022, du Teil Espina et al., 2019, Ray, 2020, Acharya et al., 2017, Sansores-Espana et al., 2021, Matteini and Florian, 2022). All of these proposed

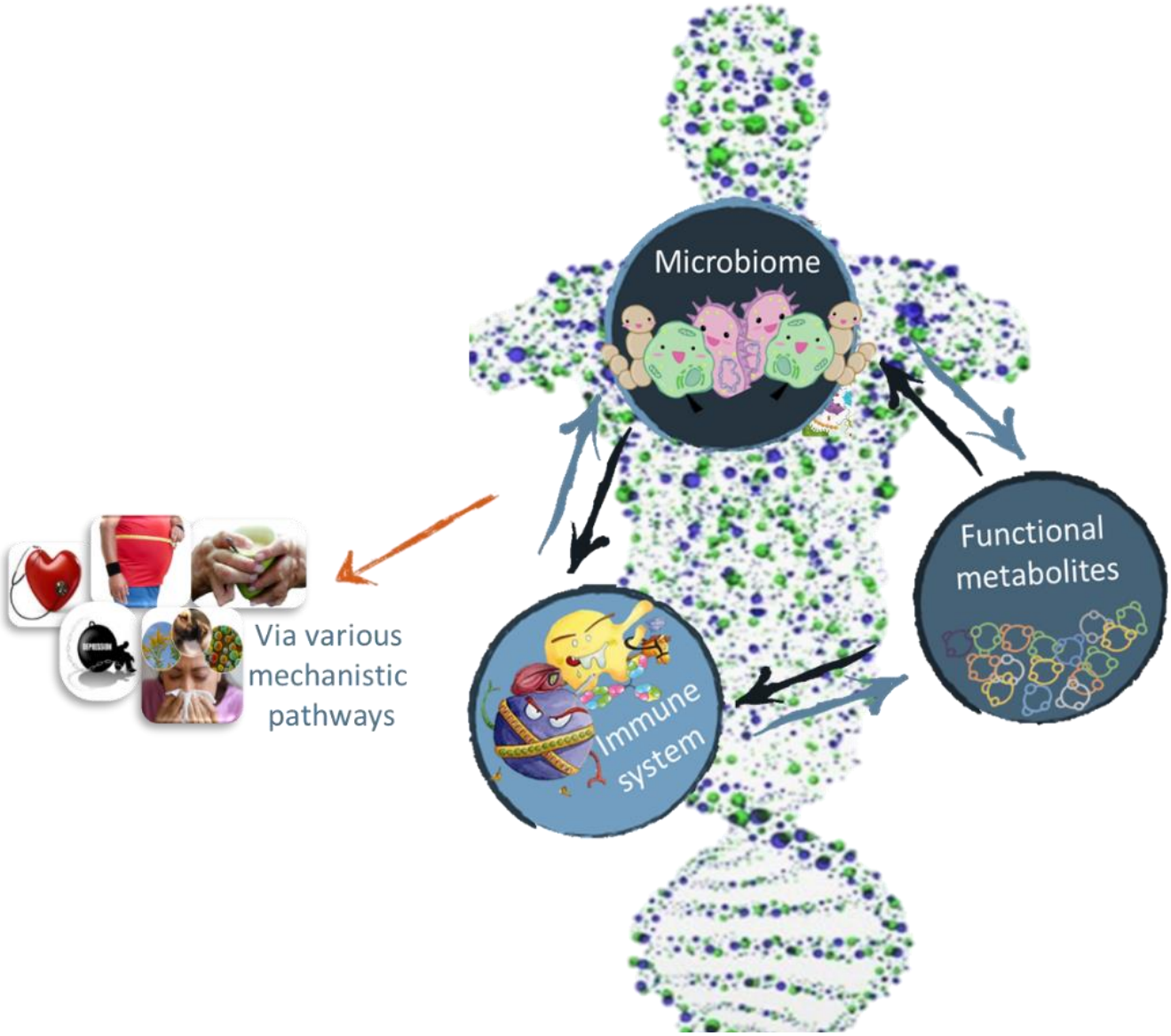

Figure 1.6. Schematic representation on how the ELA microbiome is involved in oral and systemic health

interactions dependent on different nerves for communication. Evidence show that gut linked communications pass through the vagus nerve. Nonetheless, there are not many evidence on the oral linked communications, some studies suggest the trigeminal nerve, some others the taste and olfactory receptors (Sansores-Espana et al., 2021, Dong et al., 2022, Pushpass et al., 2019). It is definitely critical to identify the exact neural pathway OM uses. Covid-19 reports have collected evidence of oral health status and a dysbiotic OM as a consequence but also as a risk factor in covid-19 symptomatology and severity (Sedghi et al., 2021, D'Amico et al., 2020, Holuka et al., 2020). In particular, loss of taste and smell symptoms of covid-19 have been associated with a particular OM profile suggesting that in the virus induced dysbiosis taste and smell receptors are utilized for communication (Xiang et al., 2020, Alsulami et al., 2023).

In summary, although oral microbiome has not been studied in extensive depth to answer mechanistic questions there are have been multiple reports from the gut microbiome (GM) being a key regulator of disease. In fact, both communities, OM and GM have the ability to communicate and interact with the host in both with direct and indirect routes. In the case of a systemic disease, the microbiome can directly interact locally with the immune cells via TLR receptors (de Aquino et al., 2014, Goncalves et al., 2016). In addition, the microbiome`s capacity to release microbial metabolites allow a distal indirect interaction with immune cell progenitors that could either strengthen or dampen the immune response via modulation of hematopoiesis (Goncalves et al., 2016, Saint-Martin et al., 2022, Trompette et al., 2014, Caffaratti et al., 2021). Any of these routes may lead to disease-onset, progression, severity while also to homeostasis. In fact, the microbiome-immune axis reveals a co-dependent interaction based on a subsequent interweave network of on/off switches capable of maintaining long term balance of systemic health. There is no doubt to this point that the OM fits well as a logical ELA-mechanism pool highlighting the need for extensive research.

1.5. Conceptualisation and proposal

After digesting the pre-existing evidence, this Thesis propose to study the adult Oral Microbiome in the context of ELA in an institutionalisation model. In this proposal, ELA phenotype is the result of long-term changes on the "immature" oral microbiome that subsequently shapes the immune phenotype together with re-activation of ELI such as CMV observed in this model. In addition, OM metabolites are contributing the shaping of the immune phenotype. Successively, both the ELA-OM metabolites and the altered immune profile lead to the ELA-phenotype.

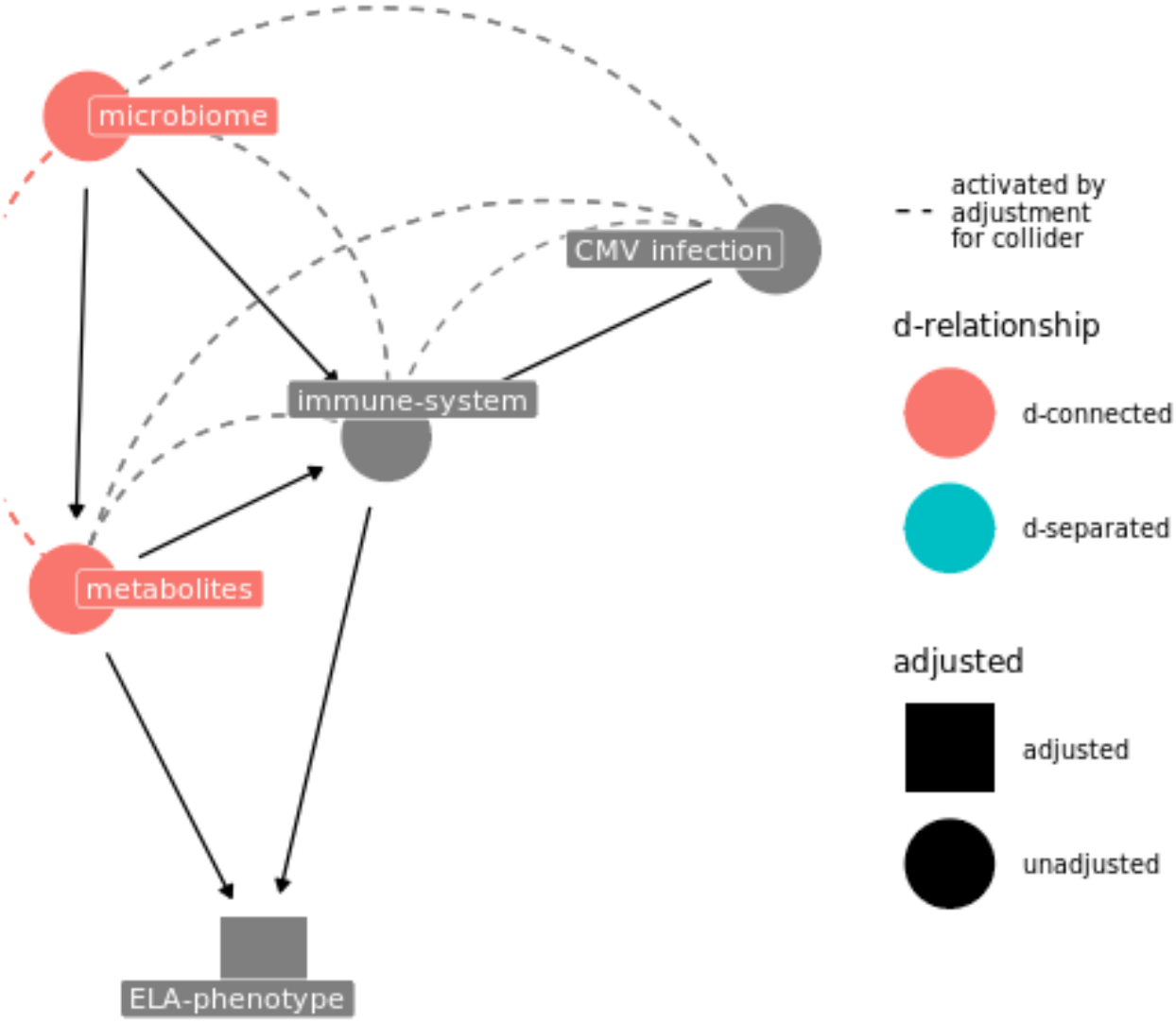

Figure 1.7. The model proposed in this Thesis represented by a Directed Acyclic Graph (DAG).

Aims and Objectives

A short narrative of the Thesis aims and objectives as addressed by each chapter

Chapter 1: Introduction of the background that this Thesis is based on and description of the components that following chapters will focus on. This Chapter is divided in 4 sections. Section 1 describes current understanding of the ELA, pre-existing literature on the ELA phenotype including immune system and psychobehavioural phenotype. Section 2 introduces the OM and how it is develops and changes from early life to later adulthood. Section 3 elucidates what are the microbial metabolites and why are these important for both host and microbes. Section 4 brings the previous section together to describe the importance of the OM as key player of oral and systemic health as well as for ELA concept. Section 5 outlines the overall aim and objectives of this Thesis.

Chapter 2: To generate evidence that the oral microbiome fits in the ELA paradigm, I first had to prove there is a relationship between the two. In this context I then hypothesised that ELA shapes the OM composition during the early life. Hence, the 1st objective was to identify imprints of ELA in adulthood. The experimental design to tackle this question was based on an institutionalisation ELA cohort, the EpiPath, in which adoptees and non-adoptees were recruited. These results were published in a data-paper titled *"Early-Life Adversity Leaves Its Imprint on the Oral Microbiome for More Than 20 Years and Is Associated with Long-Term Immune Changes"* and this Chapter reports findings of this manuscript.

Chapter 3: To generate additional evidence of the ELA-OM interaction and potential mechanism, it was indispensable to explore whether the OM had any interaction with the psychobehavioural profile of the EpiPath cohort. In this framework and guided by my earlier results showing an ELA "dysbiotic" profile I then hypothesised that the ELA-OM profile interacts with the host`s stress response. This hypothesis is covering both an observation of an existing interaction and an observation of a plausible mechanism by which ELA amends the stress response. Hence, the 2nd objective was to identify an interaction between the OM and the stress-induced cortisol and glucose measurements of the EpiPath. These results have been drafted into a manuscript format which has been submitted and is under review at the Journal of Oral Microbiology. This is a data-paper titled *"(De)Bugging stress: does the microbiome determine our reaction to a psychosocial stressor?"* and Chapter 3 describes these findings as written on this pre-submission manuscript.

Chapter 4: Following the strong observational evidence described in the previous chapters of an ELA-OM interaction, it was vital to explore what is behind this interaction in a mechanistic approach. I then hypothesised that the metabolic profile of the oral microbiome has been shaped by the experience of institutionalisation. Hence, the 3rd objective was to identify ELA marks on the metabolome of the OM in adulthood. I also aimed to identify microbial metabolites that could direct us to one or more missing mechanisms of the ELA-OM-immune system cross-talk. These results have also been drafted into a manuscript format which is currently close to submission process. This is a data-paper titled *"Early life adversity defines the metabolic capacity of the oral microbiome and associates with immune changes 20 years later."* and Chapter 4 reports these findings as described on this pre-submission manuscript.

Chapter 2. Early-Life Adversity Leaves Its Imprint on the Oral Microbiome for More Than 20 Years and Is Associated with Long-Term Immune Changes

My contribution to this Chapter: Conceptualisation; Literature review; Data generation; Data integration; Data visualisation; Final statistical analysis; Interpretation of results; Making of all figures; and Writing of the article.

This Chapter has been published as:

Charalambous, E. G., Meriaux, S. B., Guebels, P., Muller, C. P., Leenen, F. A. D., Elwenspoek, M. M. C., Thiele, I., Hertel, J. & Turner, J. D. 2021. Early-Life Adversity Leaves Its Imprint on the Oral Microbiome for More Than 20 Years and Is Associated with Long-Term Immune Changes. Int J Mol Sci, 22, 23, 12682, doi: 10.3390/ijms222312682.

This publication also includes supplementary materials that are accessible online. The electronic version of this thesis has clickable links through to them.

Abstract

The early-life microbiome (ELM) interacts with the psychosocial environment, in particular during early-life adversity (ELA), defining life-long health trajectories. The ELM also plays a significant role in the maturation of the immune system. We hypothesised that, in this context, the resilience of the oral microbiomes, despite being composed of diverse and distinct communities, allows them to retain an imprint of the early environment. Using 16S amplicon sequencing on the EpiPath cohort, we demonstrate that ELA leaves an imprint on both the salivary and buccal oral microbiome 24 years after exposure to adversity. Furthermore, the changes in both communities were associated with increased activation, maturation, and senescence of both innate and adaptive immune cells, although the interaction was partly dependent on prior herpesviridae exposure and current smoking. Our data suggest the presence of multiple links between ELA, Immunosenescence, and cytotoxicity that occur through long-term changes in the microbiome.

Keywords: early-life adversity, early experience, microbiome, bacterial community, oral microbiome, developmental origins of health and disease, immune system, host-microbe interactions

2.1 Introduction

Early-life adversity (ELA) is defined by a poor environment and conditions in early life that induce intense psychophysiological stress (Grova et al., 2019). It is mostly observed together with low socioeconomic status and is pathophysiologically correlated with a lifelong imbalance of health and disease (Turner, 2018b). The first 1000 days from conception to 2 years is the most vulnerable life period (Barker and Osmond, 1986). At birth, the body is almost fully formed; however, many biological systems continue to mature over the following years. Research on the lifelong health and disease balance has shown the significance of the environment during this period on multiple disease phenotypes (Dube et al., 2009), including cardiovascular, allergic, and autoimmune disorders, as well as mental disorders (Wampach et al., 2018, Ling et al., 2020, Reyman et al., 2019, Sarkar et al., 2021, Moore and Townsend, 2019, Backhed et al., 2015, Eriksson et al., 2014, Spitzer et al., 2013, Tomasdottir et al., 2015, Gern et al., 2009, Herzog and Schmahl, 2018, Mansuri et al., 2020). There has been a focus on the molecular mechanisms and the cellular phenotype behind the effect of stress and adversity on immune and endocrine systems as well as epigenetic modifications (Wampach et al., 2018, Elwenspoek et al., 2017b, Elwenspoek et al., 2020).

ELA has been reported to influence health trajectories via the immune system (Elwenspoek et al., 2017b, Holland et al., 2020, Reid et al., 2019), with a clear ELA-associated immunophenotype centred around the activation and functional status of T lymphocytes. In the institutionalisation model of early-life stress, strong T-cell immunosenescence has been reported (Elwenspoek et al., 2017b, Elwenspoek et al., 2017c, Reid et al., 2019). Immunosenescence is a form of accelerated immune ageing. The CD57 T- and NK- cell immunosenescence marker is absent in early life and increases with age, with high numbers of such cells in the elderly population. Immunosenescence is driven by chronic inflammation or recurrent viral infections such as CMV (Nielsen et al., 2013). NK functionality is also highly impacted by recurrent reactivation of CMV inducing NK cell exhaustion, increased cytotoxicity, and senescence (Judge et al., 2020). Additionally, such viral infections potentially program the immune system (Reid et al., 2019, Elwenspoek et al., 2017c). Latent CMV infection of haematopoietic progenitor cells reduces GR transcription and translation, impacting immune cell maturation, which can be dependent on CMV reactivation (Elwenspoek et al., 2020, Della Chiesa et al., 2012, Lopez-Verges et al., 2011).

The environment is the most critical factor shaping ELA. In the immediate postnatal period, the immune system starts maturing and the first body-area-specific microbial communities are established. Once established, the microbiome modulates the host (Yang et al., 2009), a mechanism to protect symbiotic microbial communities, where cases of microbial dysbiosis can be fatal (Wang et al., 2020). The ELM plays important roles in an infant's subsequent development (Wampach et al., 2018, Shao et al., 2019) and a long-term health trajectory (Shao et al., 2019, Wampach et al., 2018, Sarkar et al., 2021, Yang et al., 2016, Moore and Townsend, 2019, Backhed et al., 2015). Nevertheless, the development of the ELM is critically impacted by the environment, mode of birth, early-life nutrition, and environmental exposure, which leave a clear lifelong trace (Herzog and Schmahl, 2018, Miller et al., 2018). For example, maternal milk is rich in essential nutrients, protective antibodies, and components essential for the developing microbiome, such as human milk oligosaccharides (HMOs) and short-chain fatty acids (SCFAs) (Zijlmans et al., 2015, Alcon-Giner et al., 2020, Stewart et al., 2018, Backhed et al., 2015, Toussaint et al., 2016, Duran-Pinedo et al., 2018).

The oral microbiome (OM) is composed of various distinct, smaller communities within the oral cavity (Carpenter, 2020, Boustedt et al., 2015, Chu et al., 2017, Mason et al., 2018, Mark Welch et al., 2019) that are robust, stable, and particularly resilient (David et al., 2014, Shaw et al., 2017), particularly to antibiotic therapy (Shaw et al., 2017, Marsh, 2006, Zaura et al., 2015, Kennedy et al., 2019, Abeles et al., 2016). Moreover, saliva contains actively secreted components such as cortisol, glucose, lactate, urea, and proteins, such as polypeptides, glycoproteins (cystatins, mucins, and immunoglobulins) and antimicrobial peptides (histatins, defensins, and immunoglobulins-IgA). Many of these are energy sources for the OM, and salivary glycoproteins are the principal nutrient source. These substrates are crucial for the development of multispecies communities and their preservation (Carpenter, 2020, Kennedy et al., 2019, Almeida-Santos et al., 2021), and enhance the resistance of the community to environmental stressors (Carpenter, 2020, Mukherjee et al., 2021, Jakubovics, 2015). The long-term stability of the OM leads to the hypothesis that, once established in early life, it remains stable, robust, and resilient, retaining an imprint of the early environment (David et al., 2014, Shaw et al., 2017).

We previously reported higher virally mediated activation and senescence of the immune system in association with ELA in the EpiPath cohort (Elwenspoek et al., 2017a,

Elwenspoek et al., 2017c). This cohort consists of young adults exposed to ELA by either institutionalisation or separation from their parents at birth, and were subsequently adopted into Luxembourg, while controls were raised by their biological parents. With the growing evidence of a microbiome–immune–system interaction, we attempted to identify if institutionalisation left a mark on the OM of the adoptees. In this study, we sequenced the 16s-rRNA from the buccal and salivary bacterial communities from our cohort. Integrating this with the full immunophenotype, we identified associations with various taxa and analysed how the microbiome interacts with the immune system.

2.2 Results

The V4 region of the bacterial 16S gene was successfully amplified from both buccal swabs and salimetrics oral swabs for the 115 members of the EpiPath cohort, and a total of 45 Gbp sequencing data were obtained. All samples were successfully processed using mothur and a total of 288 and 371 genera from 24 phyla were identified for buccal and salivary samples respectively. The saliva and buccal swabs from the EpiPath cohort were examined independently as they are two distinct oral communities from the same participants.

2.2.1 Microbial Diversity and Overall Microbial Composition

2.2.1.1 Salivary Microbiome (α- and β-Diversity)

We identified sequences from all of the 24 principal bacterial phyla in the overall salivary microbial community. Within these 24 phyla, the most abundant in both control and ELA groups were *Actinobacteria, Proteobacteria, Firmicutes,* and *Fusobacteriota* (Figure 1A). The most *abundant genera of the salivary composition were Acinetobacter, Micrococcaceae, Actinobacillus, Rothia, Corynebacterium, Micrococcus, Actinomyces, Alloprevotela, Porphymonas, Fusobacterium, Weeksellaceae, Flavobacterium, Bradyrhizobium, Porphyromonas, _ Comamonas, Olsenella, Fluviicola, Fusobacterium, Absconditabacteriales _(SR1)_ge, Streptobacillus, Fretibacterium, JGI_0000069-P22_ge, Capnocytophaga, Pseudarcicella, Tannerella, Prevotella,* and *Campylobacter* (Figure 1B). There was no difference in α-diversity between the controls and the adoptees in terms of diversity (controls: mean = 13.87872, SD = 7.127721, adoptees: mean = 14.14869, SD = 6.601899, Wilcoxon rank sum test p = 0.7579) and evenness (controls: mean = 0.5619667, SD = 0.06864806, adoptees: mean = 0.5646776, SD = 0.06172601, Wilcoxon rank sum test

p = 0.9461). Plotting the Shannon evenness index against the inverse Simpson diversity index confirmed that there analogous diversity and evenness between the controls and adoptees (Supplementary Figure S1). Principal coordinate analysis could not detect systematic differences either (Supplementary Figure S1).

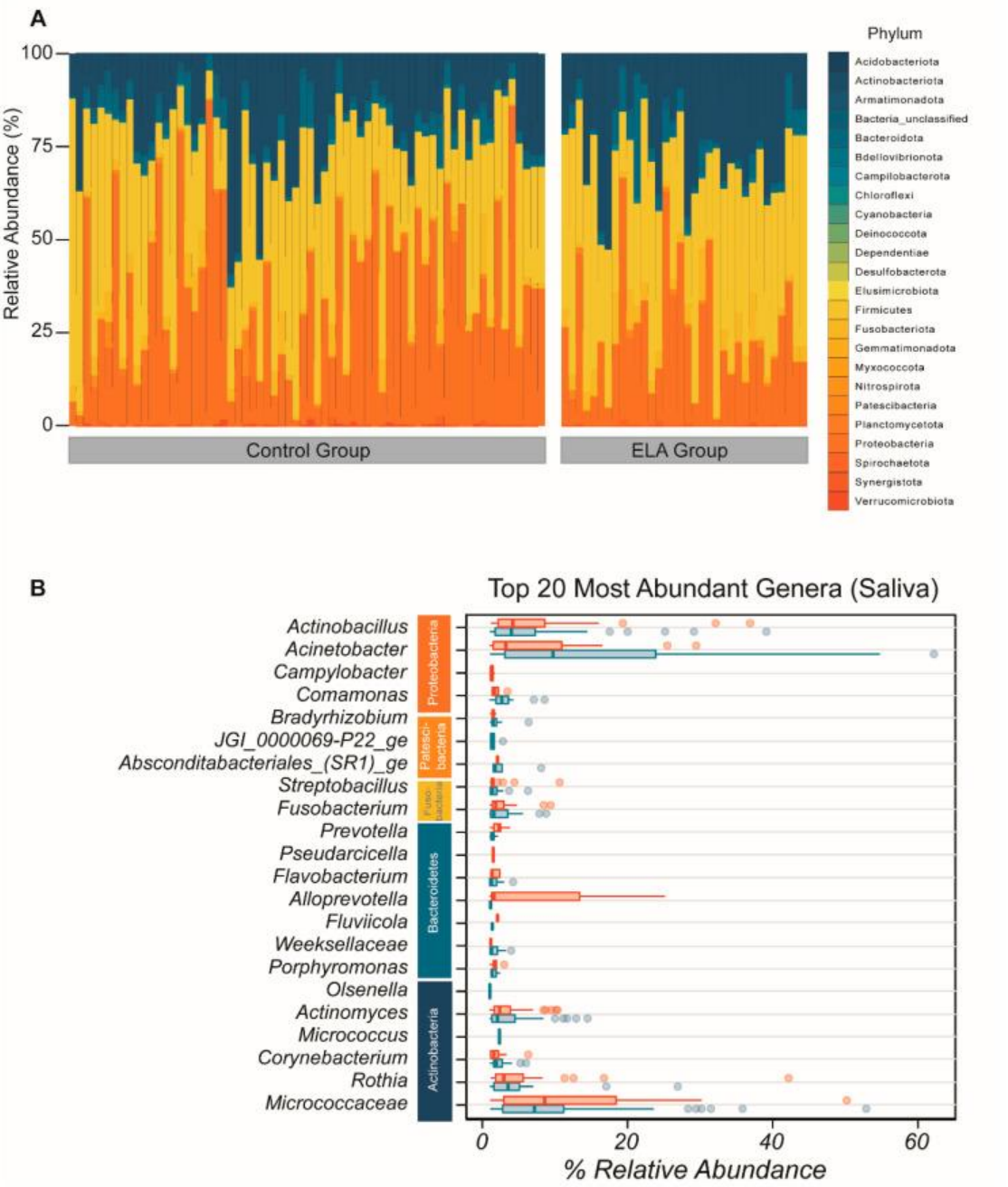

Figure 2.1. Overall composition of salivary bacterial community. (**A**) Overall microbial composition displayed in stacked area bar plot with the percentage relative abundance of all phyla found in each participant in both study arms. (**B**) Top 20 most abundant genera by mean abundance arranged graphically by phyla. Vertical line = mean; rectangle = 1st to 3rd quartile; horizontal lines = 2.5th to 97.5th percentile. Outliers are indicated as individual data points. Blue, control group; red, ELA group.

2.2.1.2. Buccal Microbiome (α- and β-Diversity)

As for the salivary microbiome, we identified sequences from all the 24 principal bacterial phyla in the buccal microbial community. The most abundant phyla were the same as in the salivary microbiome: *Actinobacteria*, *Proteobacteria*, *Firmicutes*, and *Fusobacteriota* (Figure 2A). The most abundant genera were *Actinomyces*, *Corynebacterium*, *Micrococcaceae*, *Rothia*, *Alloprevotela*, *Porphymonas*, *Fusobacterium*, *Actinobacillus*, *Bradyrhizobium*, *Haemophilus*, *Methylobacterium-Methylorubrum*, *Oxalobacteracea*, *Actinomyces*, *Neisseria*, *Paucibacter*, *Lautropia Cardiobacterium*, *Brucella*, *Alysiella*, and *Campylobacter*, therefore revealing a substantial overlap in the detected genera between the saliva and the buccal microbiome (Figure 2B).

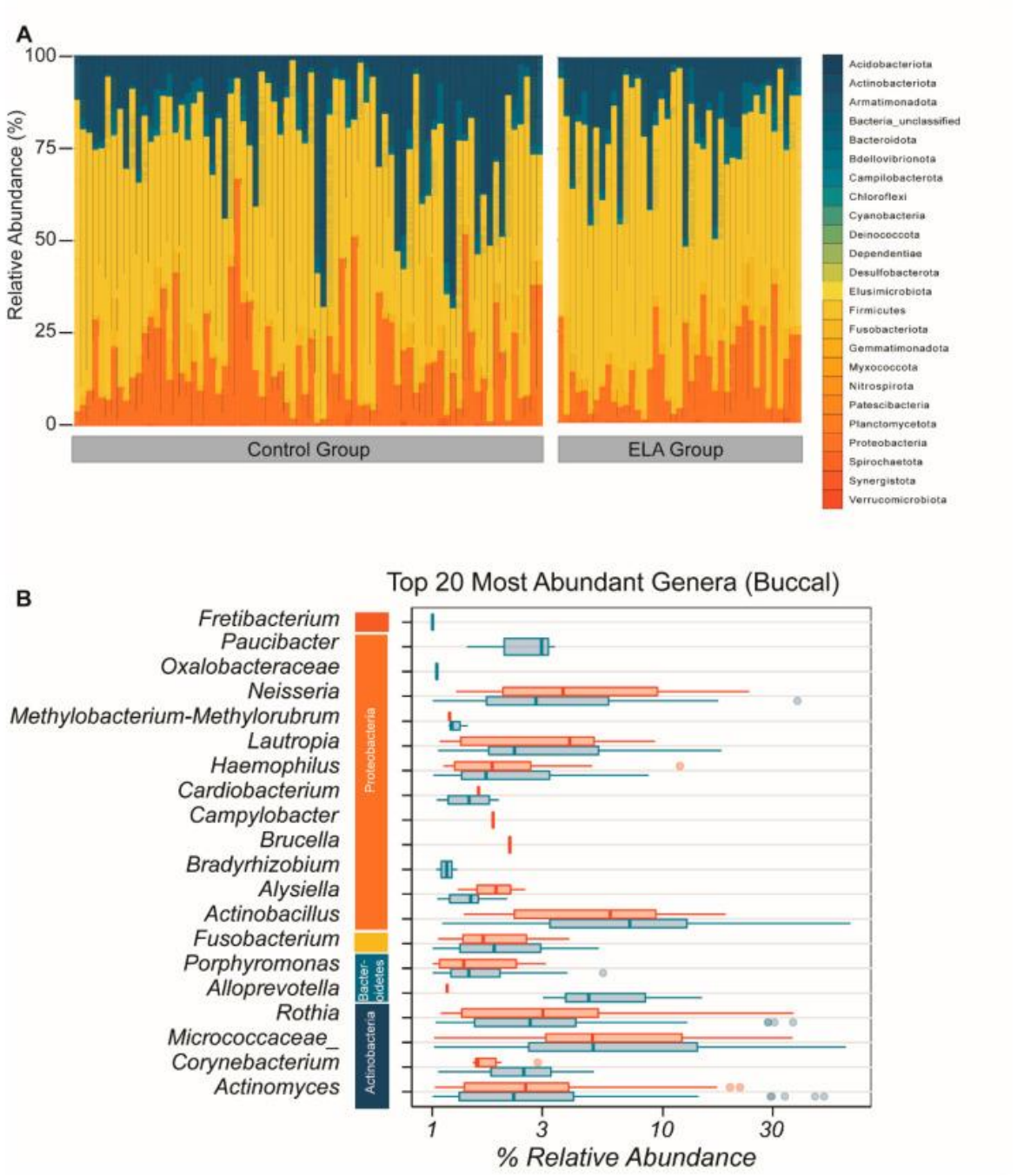

Figure 2.2. Overall composition of the buccal bacterial community. (**A**) Overall microbial composition displayed in stacked area bar plot with the percentage relative abundance of all phyla found for each participant in both study arms. (**B**) Top 20 most abundant genera by mean abundance arranged graphically by phyla. Vertical line = mean; rectangle = 1st to 3rd quartile; horizontal lines = 2.5th to 97.5the percentile. Outliers are indicated as individual data points. Blue, control group; red, ELA group.

As for the salivary microbiome, we observed a very similar diversity and evenness between controls and adoptees as measured by the inverse Simpson index (controls: mean = 12.85828, SD = 10.117292, adoptees: mean = 14.72315, SD = 8.991065, Wilcoxon rank sum test p = 0.08803). The Shannon evenness index was similar between adoptees and controls, and, in both, it was higher than in the salivary microbiome (controls: mean = 0.5137724, SD = 0.10141122, adoptees: mean = 0.465861, SD = 0.08482336, Wilcoxon rank sum test p = 0.09024). α-diversity was again similar in the controls and the adoptees in terms of evenness (Wilcoxon rank sum test p = 0.08803) and diversity (Wilcoxon rank sum test p = 0.09024). Plotting the Shannon evenness index against the inverse Simpson diversity index revealed no systematic differences in diversity or evenness between the controls and adoptees (Supplementary Figure S1). Similarly, principal coordinate analysis indicated no differences between adoptees and control (Supplementary Figure S1).

2.2.1.3. Salivary and Buccal Microbiomes Are Two Separate Entities

To ensure that sample collection was performed correctly and that we had two distinct communities, we compared the diversity and evenness of the salivary and buccal samples. We found a low correlation between the salivary and buccal communities in both the inverse Simpson diversity index (Figure 3A, p = 0.47, rho = −0.07372058, Spearman's rank correlation test) and Shannon evenness index (Figure 3B, p = 0.8759, rho = 0.01595802, Spearman's rank correlation test), giving evidence in favour of the hypothesis that, despite their close physical proximity, they can be seen as distinct communities. Comparing the salivary and buccal microbiomes by group, the diversity ratios of the salivary against buccal communities were similar between the controls and adoptees (Figure 3A, controls: p = 0.3311, rho = −0.1222465; adoptees: p = 0.7898, rho = 0.04812834; Spearman's rank correlation test, Figure 3B; controls: p = 0.9578, rho = 0.009692513; adoptees: p = 0.7898, rho = −0.04812834; Spearman's rank correlation test). This suggests that the overall composition between the controls and adoptees may be similar, but differences would be seen at the phyla level.

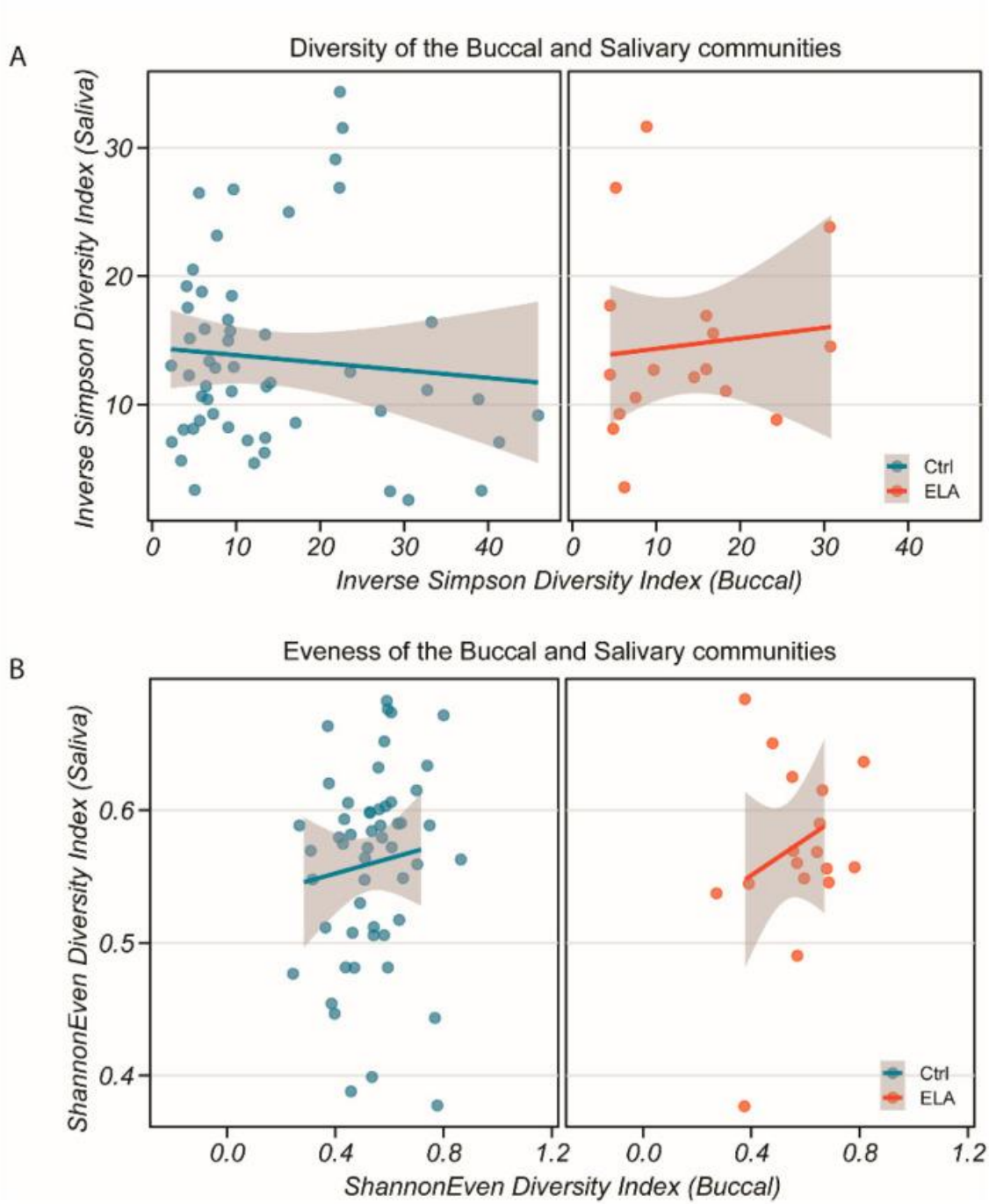

Figure 2.3. Diversity and evenness of the salivary and buccal bacterial communities in both study groups. (**A**) Inverse Simpson diversity index of saliva against buccal communities for control (**left**) and ELA (**right**). (**B**) Shannon evenness index of saliva against buccal communities for control (**left**) and ELA (**right**). No correlation was found between either community for either measure or group (Spearman's rank correlation test, $p > 0.47$). Grey shaded area: 95% confidence interval.

2.2.1.4. ELA Induces Differences in Specific Taxa in Both Salivary and Buccal Communities

Investigating the abundance levels of phyla and genera highlighted differences in the community composition across the ELA group and healthy controls (Table 1). While there were no differences in the phyla level in the buccal data, *Proteobacteria* and *Verrucomicrobiota* were significantly lower (FDR < 0.05 for both) in the adoptees in comparison to the controls (Supplementary Table S1A, Figure 4A,B) in the saliva microbiome as detected in fractional regression analyses. Analyses of deeper taxonomy revealed two of the most abundant genera of the *Proteobacteria* phylum, *Comamonas* and *Acinetobacter*, to be significantly lower in the saliva of adoptees compared to controls alongside *Aquabacterium* and unclassified *Comamonadaceae* (Table S1B, Figure 4C–F). In conclusion, while we could not detect systematic differences in the buccal microbiome between the ELA group and the controls, the saliva microbiome was structurally different in its composition, with a prominent role for *Proteobacteria* genera (Figure 4A).

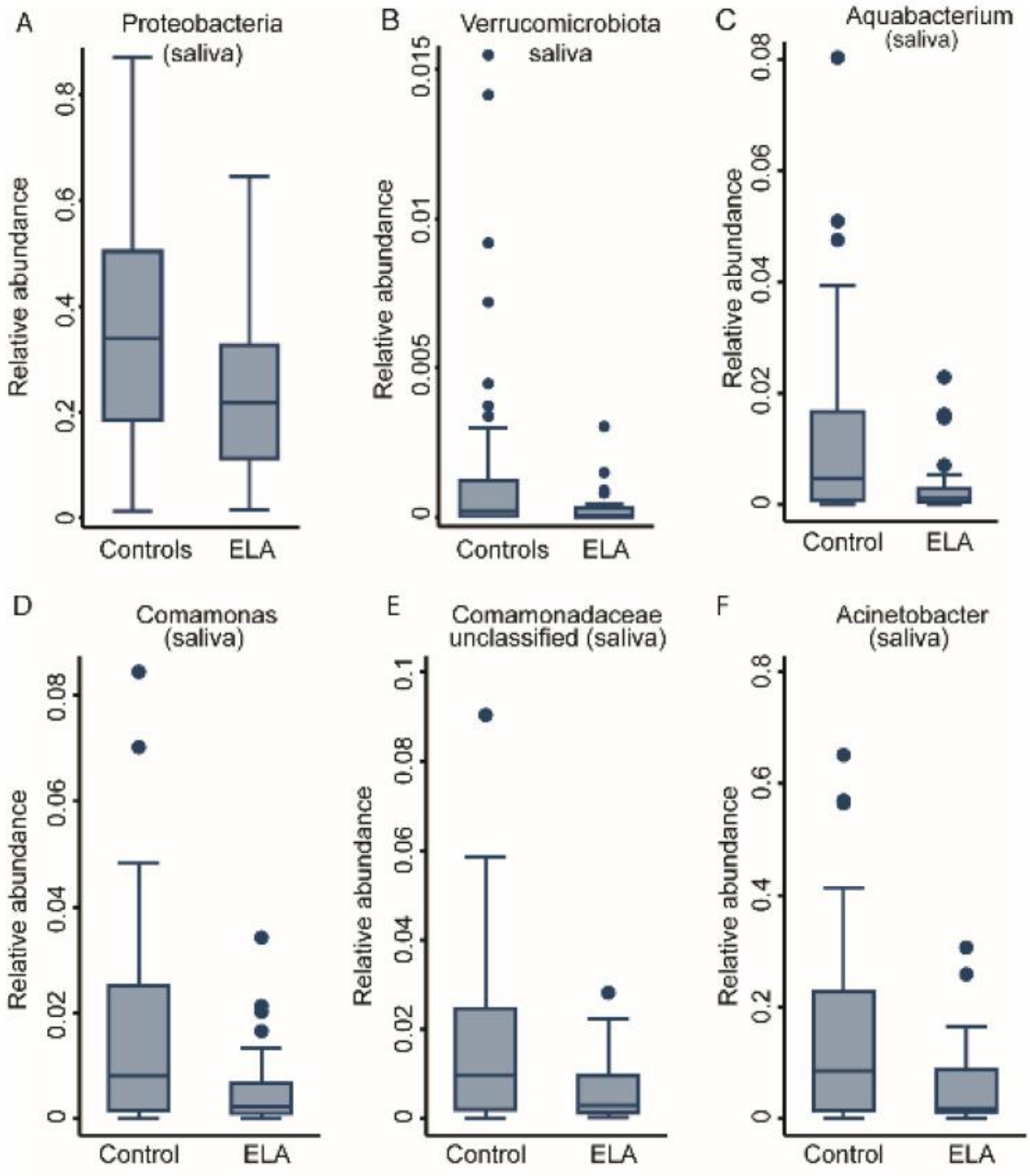

Figure 2.4. Taxonomic differences between study groups in the salivary bacterial community. Box plots of two phyla, (**A**) Proteobacteria and (**B**) Verruomicrobiota, as well as four genera: (**C**) Aquabacterium, (**D**) Comamonas, (**E**) Comamonadaceae, unclassified and (**F**) Acinetobacter. All are significantly associated with study group; fractional regressions against study group were calculated to determine significance (FDR < 0.05). Horizontal line = mean; rectangle = 1st to 3rd quartile; vertical lines = 2.5th to 97.5th percentile. Outliers are indicated as individual data points. Blue, control group; red, ELA group.

2.2.3. Environmental Covariates

Next, we investigated the impact of environmental factors on the OM to potentially explain the effects of ELA described above (Table 1).

Table 2.1. An overview of the bacterial taxa associated with all the tested covariates in both saliva and buccal microbiomes.

	Saliva			Buccal		
	Number of Associations (FDR < 0.05)	**Positively Associated Taxa**	**Negatively Associated Taxa**	**Number of Associations (FDR < 0.05)**	**Positively Associated Taxa**	**Negatively Associated Taxa**
Smoking	0	-	-	5	-	*Pasteurellaceae* (unclassified), *Neisseria*, *Capnocytophaga*, *Neisseriaceae* (unc [1]), *Planococcaceae* (unc)
ELA vs. control	4	-	*Aquabacterium*, *Comamonas*, *Comamonadaceae* (unc), *Acinetobacter*	0	-	-
Anti-CMV seropositive	2	-	*Pseudomonas*, *Oxalobaceraceae* (unc)	9	*Alysiella*, *Neisseria*	*Sphingomonas*, *Acinetobacter*, *Oxalobacteraceae* (unc), *Bradyrhizobium*, *Flavobacterium*, *Methylorubrum*, *Comamonadaceae* (unc)
Anti-EBV seropositive	0	-	-	1	*Neisseria*	-
HSV	0	-	-	0	-	-
CD4+ CD57+	2	*Selenomonas*	*Oxalobaceraceae* (unc)	4	*Selenomonas*, *Capnocytophaga*, *Campylobacter*, *Lautropia*	-
CD8+ CD57+	0	-	-	0	-	-
Total CTLs	0	-	-	0	-	-
Total T_h cells	0	-	-	0	-	-

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[1] unc = unclassified.

2.2.3.1. Smoking

As lifestyle has a pivotal role in the development of the microbiome, we assessed the effect of smoking on the OM by including smoking status (binary: smokers vs. non-smokers) into the regression modelling (Table 1). No significant genera were detected for the salivary community, whereas from the buccal community, we exposed five genera negatively

associated with smoking: three from the *Proteobacteria* phylum, *Neisseria*, *Neisseriaceae_unclassified*, and *Pasteurellaceae_unclassified*; 1 from the *Bacteroidetes* phylum and *Capnocytophaga* genus; as well as one from the *Firmicutes* phylum and *Planococcaceae_unclassified* genus (FDR < 0.05, Figure 5A–E). In sensitivity analyses, we removed smoking as a covariate from the regression equations for the FDR-corrected significant genera to explore potential effect mediation through smoking, but the results remained virtually unchanged.

The full results for the buccal and saliva microbiomes can be found in Supplementary Table S2.

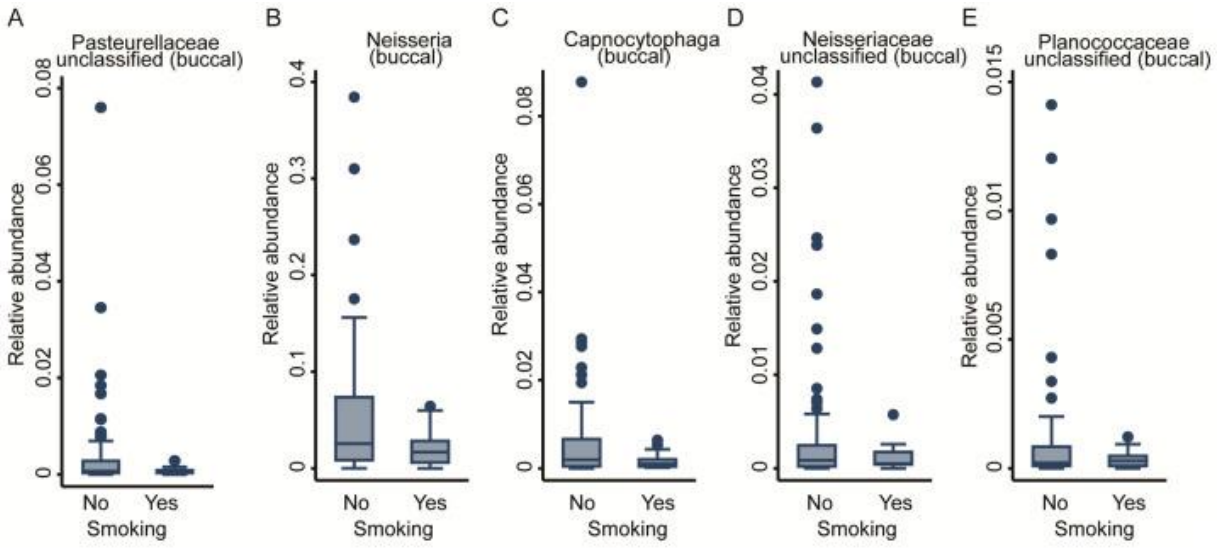


Figure 2.5. Taxonomic differences in the buccal bacterial community associated with smoking. Box plots of five genera: (**A**) Pasteurellaceae unclassified, (**B**) Neisseria, (**C**) Capnocytophaga, (**D**) Neisseriaceae unclassified, and (**E**) Planococcaceae unclassified. All are significantly associated with study group; fractional regressions against smoking were calculated to determine significance (FDR < 0.05). Horizontal line = mean; rectangle = 1st to 3rd quartile; vertical lines = 2.5th to 97.5th percentile. Outliers are indicated as individual data points.

2.2.3.2. Prior Viral Infections

We previously reported that viral infections may mediate the early-life immunophenotype (Elwenspoek et al., 2017c). Consequently, we tested whether prior viral infection, measured as anti- HSV, EBV, and CMV seropositivity, affected the oral bacterial communities. We achieved this via fractional regressions with the antibody titre (binary: positive vs. negative) as the predictor of interest and the genus abundance as the response variable (Table 1). Due to low case numbers of positive titre results for CMV, we could not adjust for basic covariates without inducing numerical instability in the fitting procedure. While HSV titres did not show any association, a positive EBV titre was positively associated with the abundance of the Neisseria genus in the buccal microbiome (FDR < 0.05, Figure 6A). However, anti-CMV antibody titres showed a very strong association with the OM. In total, 10 genera had a significant association with CMV titres. Nine genera from the Buccal community, eight derived from the Proteobacteria phylum (Acinetobacter, Bradyrhizobium, Comamonadaceae_unclassified, Methylobacterium-Methylorubrum, Oxalobacteraceae_unclassified, and Sphingomonas genera) unveiled a negative association, whereas the genera of Alysiella and Neisseria demonstrated a positive association. One genus from Bacteroidetes phylum, Flavobacterium, also appeared to be negatively associated with positive CMV titres (FDR < 0.05, Figure 6B–J). Two genera of the Salivary community from the Proteobacteria phylum, Pseudomonas and Oxalobaceraceae_unclassified, exhibited a negative association (FDR < 0.05, Figure 7A,B). The full results for the buccal and the saliva microbiome antibody titre associations can be found in Supplementary Table S3. In a further step of sensitivity analysis, we included a positive antibody titre as a covariate into the regression models to investigate the differences in the genus abundances between ELA and controls. However, the results virtually remained the same, indicating either insufficient statistical power to detect potential mediation or that CMV exposure does not mediate ELA-related changes in the OM. This suggests that, unlike increased immunosenescence, the changes we saw in the oral bacterial community are independent of prior exposure to Herpesviridae.

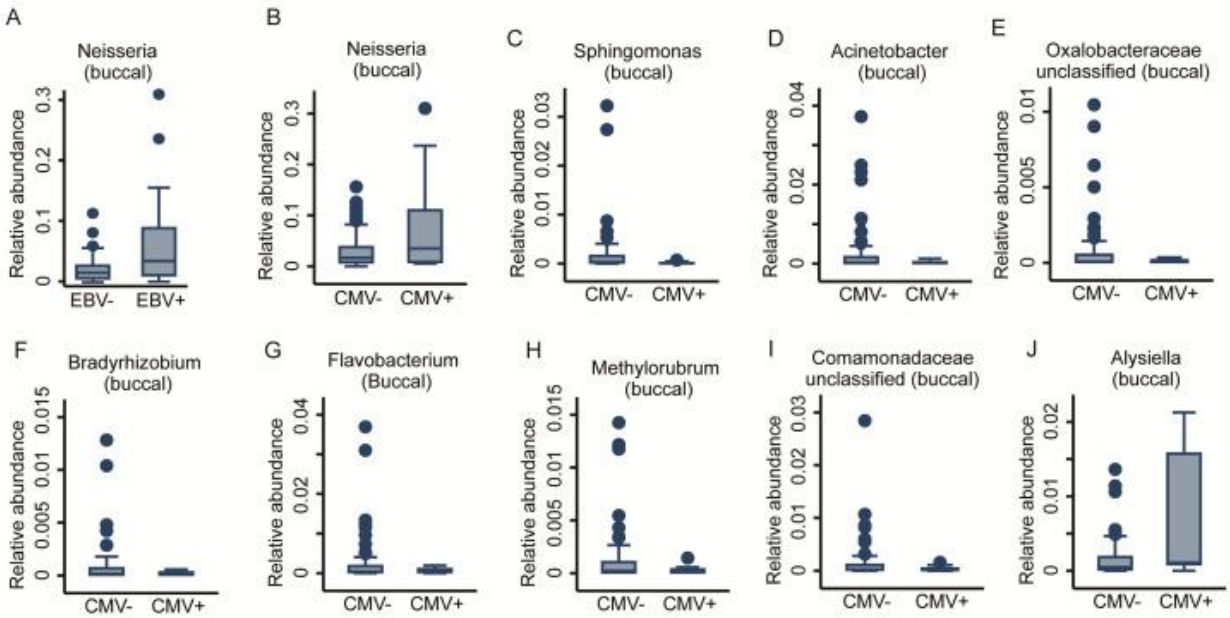


Figure 2.6. Taxonomic differences in the buccal bacterial community associated with anti-*herpesviridiae* serological status. Box plots of one genus (**A**) *Neisseria* significantly associated with anti-EBV antibody titres. Nine genera, (**B**) *Neisseria*, (**C**) *Sphingomonas*, (**D**) *Acinetobacter*, (**E**) *Oxalobacteraceae unclassified*, (**F**) *Bradyrhizobium*, (**G**) *Flavobacterium*, (**H**) *Methylorubrum*, (**I**) *Comamonadeceae unclassified*, and (**J**) *Alysiella*, were significantly associated with anti-CMV antibody titres. Fractional regressions against the presence of anti-EBV and anti-CMV antibodies were calculated to determine significance (FDR < 0.05). Horizontal line = mean; rectangle = 1st to 3rd quartile; vertical lines = 2.5th to 97.5th percentile. Outliers are indicated as individual data points.

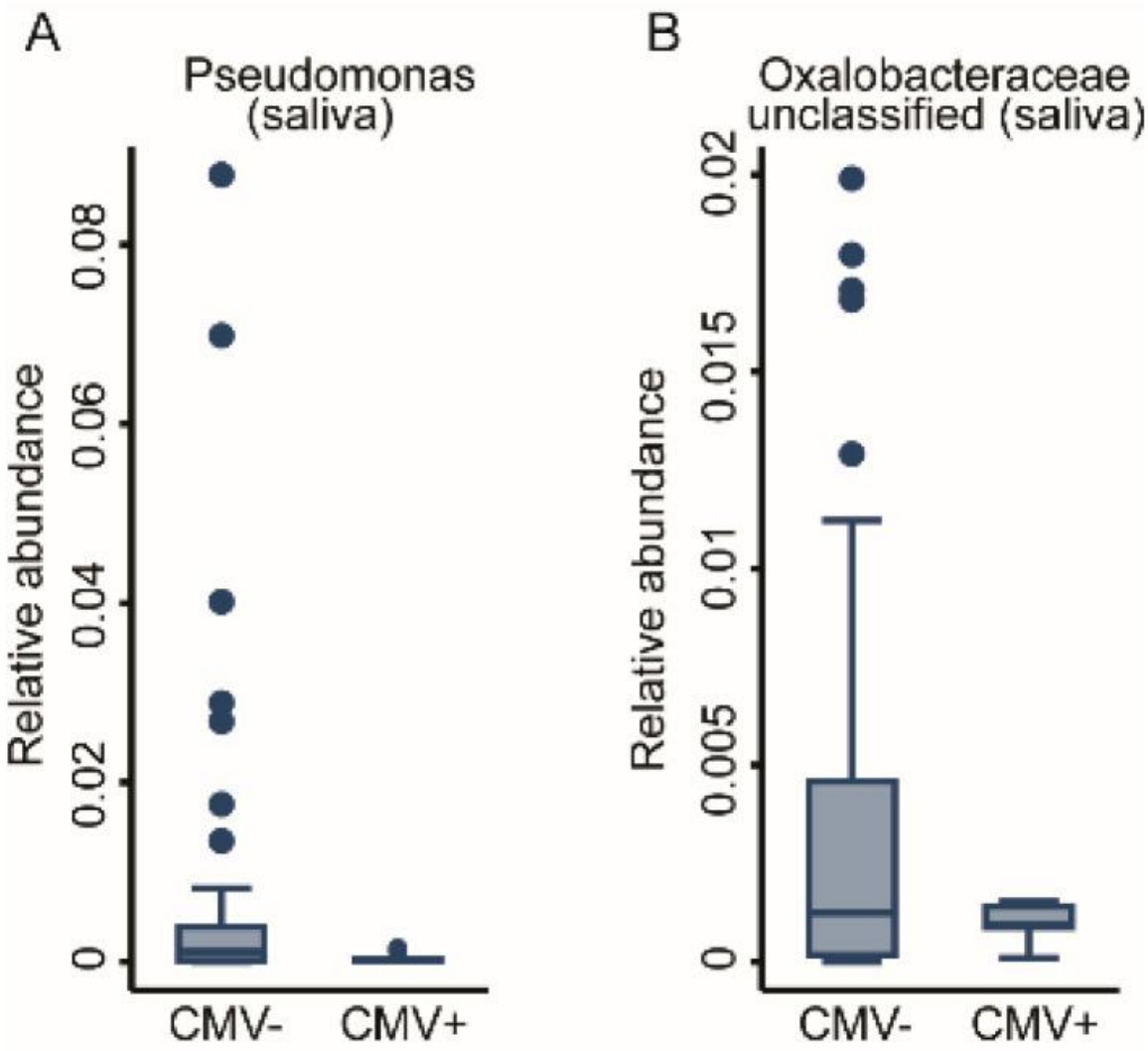


Figure 2.7. Taxonomic differences in the salivary bacterial community associated with anti-CMV serological status. Box plots of two genera, (**A**) *Pseudomonas* and (**B**) *Oxalobacteraceae unclassified*, which were significantly associated with CMV antibody titres. Fractional regressions against the presence of anti-CMV antibodies were calculated to determined significance (FDR < 0.05). Horizontal line = mean; rectangle = 1st to 3rd quartile; vertical lines = 2.5th to 97.5th percentile. Outliers are indicated as individual data points.

2.2.4. Fractional Regression Models of the Immune–Microbiome Interactions

In the next step, we fitted a series of fractional regression models integrating the relative abundance of the taxonomic levels in the salivary and buccal compositions with our previously published immune-system profiling. Among the full dataset of 48 immune cell populations, we identified 11 significant associations with genera, most importantly for T cells and NK cells (Table 1).

2.2.4.1. Association with CD4 T-Cell Immunosenescence

Immunosenescence is a common result of adversity. Thus, we decided to look for possible associations between adversity, microbiome, and accelerated ageing of immune cells (Table 1). For screening the OM associations with the share of CD57 positive CD4 and CD8 cells, we used multivariable fractional regressions including the genus abundance as the response variable, and the share of CD57-positive CD4 and CD8 cells as a predictor of interest and the basic set of covariates. Additionally, we included the study-group variable as a covariate to control for potential confounding factors related to ELA status. CD8 T cells were previously reported to be significantly associated with CMV (Elwenspoek et al., 2017c), but we found no associated taxonomic markers from the OM. From CD4 T-cells tests, we identified six strong taxonomic associations. Two genera from the salivary microbiome, *Selenomonas* from the *Firmicutes* phylum showed a positive association and *Oxalobacteraceae_unclassified* from the *Proteobacteria* phylum showed a negative association. Four genera from the buccal community: *Selenomonas* from *Firmicutes*, *Capnocytophaga* from the *Bacteroidetes* phylum, and *Campylobacter* and *Lautropia* from the *Proteobacteria* phylum, displayed a positive association (FDR < 0.05, Figure 8A–F). For further exploration, we fit additional fractional regressions using the number of T-helper cells and T-killer cells as predictors of interest using the same set of covariates as before, finding no additionally significant associations after correction for multiple testing. Summary statistics for the buccal and saliva microbiomes are given in Supplementary Table S4.

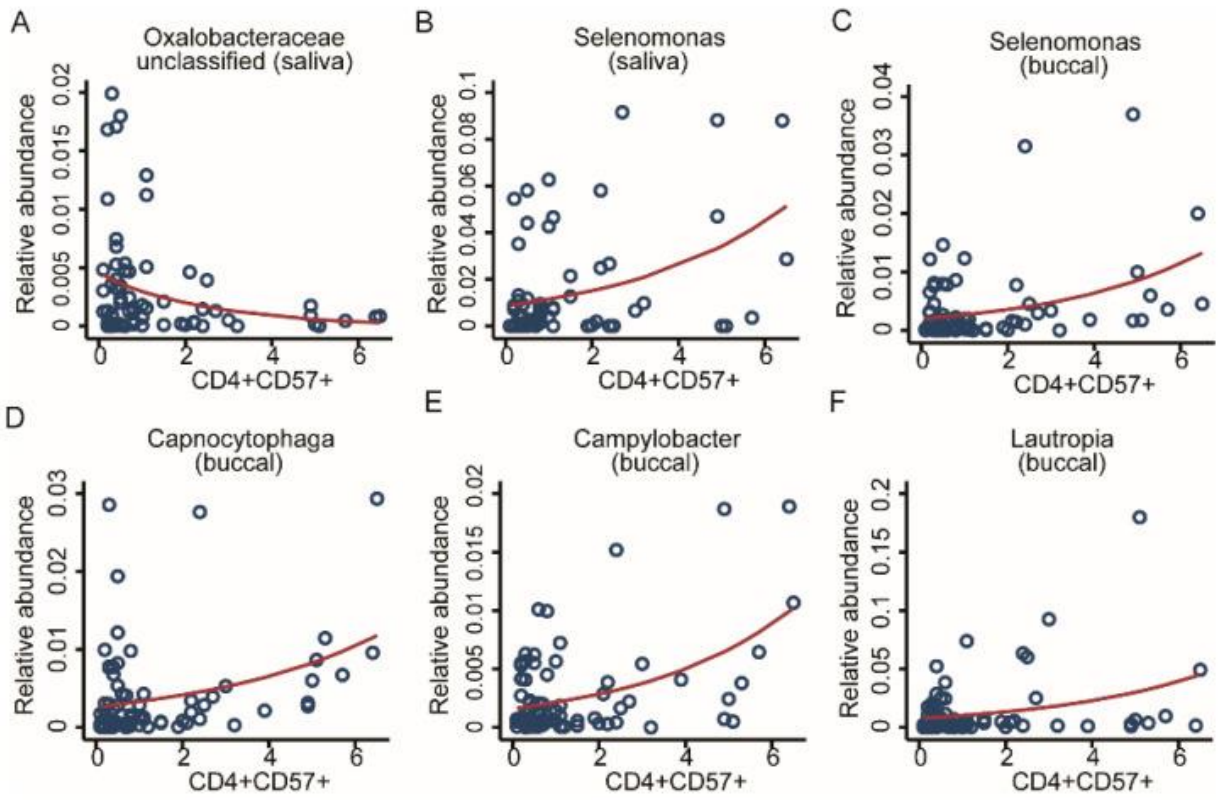


Figure 2.8. Taxonomic associations in both communities with immunosenescence. Scatter plots with regression lines for six genera. From the salivary community, two genera, (**A**) *Oxalobacteraceae unclassified* and (**B**) *Selenomonas*, as well as four genera from the buccal community, (**C**) *Selenomonas*, (**D**) *Capnocytophaga*, (**E**) *Campylobacter*, and (**F**) *Lautropia*, were all significantly associated with CD4 CD57 cell counts. Fractional regressions against CD4 CD57 cell counts were calculated to determine significance (FDR < 0.05). Regression lines were derived from fractional regressions with logistic parametrisation of the conditional mean.

2.2.4.2. Association with NK Cell Activity

Innate immune cells such as natural killer (NK) cells are the first line of defence and often interact with commensal bacteria. Adoptees of this cohort showed increased cytotoxicity on their NK cells (Fernandes et al., 2021); hence, we thought to assess for a potential link with the microbiome. Through screening the OM for associations with various types of NK cells, we found three genera associated with cell counts with an FDR < 0.05, while seven additional associations reached an FDR < 0.1 (Supplementary Table S4), hinting that a better-powered study may find a broader association pattern. In the buccal community, the Oribacterium genus showed a negative association with the total number of NK cells and the total number of mid-maturation NK cells (FDR < 0.05, Figure 9A,B). In parallel, within the salivary community, several genera were significantly associated with different stages

of NK maturation. Pseudomonas was found to be positively associated with the total number of CD25 expressing NK cells, which reflects an association with the global activation of NK cells (FDR < 0.05, Figure 9C). The abundance of Alloprevotella was positively associated with the abundance of activated immature CD25CD56hi expressing NK cells (FDR < 0.05, Figure 9D).

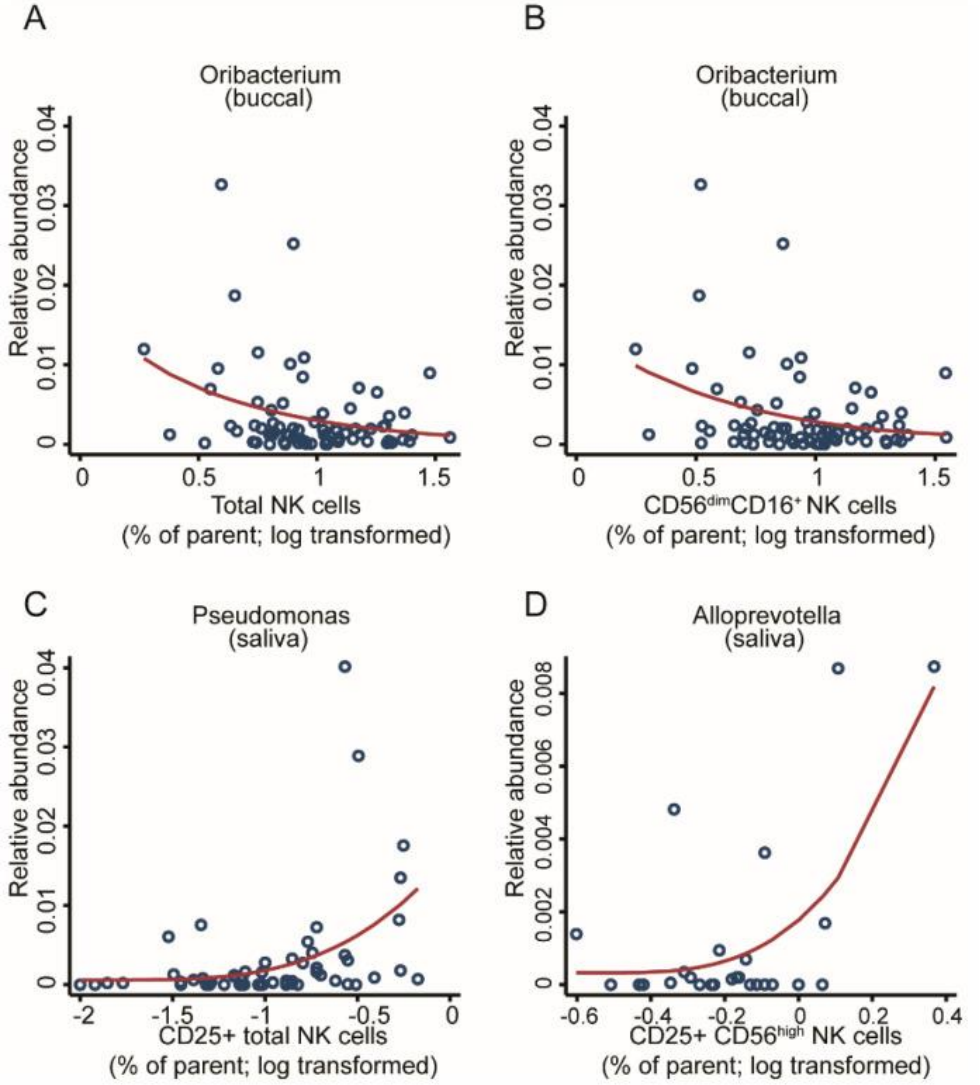


Figure 2.9. Taxonomic associations in both communities with natural killer cell activity. Scatter plots with regression lines for one genus from the buccal community, *Oribacterium*, was significantly associated with (**A**) the total number of NK cells and (**B**) the total number of mid-maturation NK cells as well as two genera from the salivary community: (**C**) *Pseudomonas*, associated with $CD25^{+}$ NK cell counts; and (**D**) *Alloprevotella*, associated with $CD25CD56^{hi}$ NK cell counts. Fractional regressions against NK cell counts were calculated to determine significance (FDR < 0.05). Regression lines were derived from fractional regressions with logistic parametrisation of the conditional mean.

Summary statistics for the buccal and saliva microbiome are given in Supplementary Table S4 and bacterial taxa are highlighted in Table 1.

2.3. Discussion

In this study, we identified taxonomic differences in the OM 24 years after adversity that were common throughout a cohort of diverse cultural and ethnic origins. We identified genera that had a significantly reduced abundance in the adoptees, which were significantly associated with smoking; immunosenescence of CD4 T cells; circulating number and activation status of NK cells; and anti-CMV and, to a lesser degree, anti-EBV titres. Importantly, we were able to see these differences in both the salivary and buccal microbiomes, both of which are readily accessible and both are regularly and easily sampled, even if the buccal microbiome is somewhat underexplored to date. Our data highlight the distinctness of the salivary and buccal microbiomes in distinct oral niches with unique microbial signatures.

Our findings from the EpiPath cohort closely mirror those of Reid et al. (Reid et al., 2021), although in significantly different microbial communities. We report differences in the abundance of taxa associated with early institutionalisation and CMV seropositivity. Considering that the gut microbiome (GM) is far more labile to lifestyle and environmental impact than the OM (Reid et al., 2021), our findings build upon those of Ried et al., opening the possibility of much longer-term studies, as the enhanced stability of the OM suggests that differences may be stable over many decades (Reid et al., 2021). Expanding our analyses to associations with the immunosenescent CD4 T cells and the activation status of circulating NK cells strengthens the possible role of microbe–immune cross-talk in ELA and the potentially detrimental outcomes. Furthermore, at the family taxonomic level, we observed highly similar differences to those reported by Reid et al. (e.g., *Prevotella* vs. *Alloprevotella*, both from the *Prevotellaceae* family). This highlights the link between the oral and GMs, as numerous studies provide evidence of bacteria migrating from the oral cavity and colonising the gut, whereas there is no evidence of the opposite happening (Prodan et al., 2019, Valdes et al., 2018, Ridlon et al., 2014, Schmidt et al., 2019).

Our current findings show that institutionalised, genetically unrelated individuals share particular taxa, identifiable 24 years later, independent of the event of adoption. The buccal community, in contrast to the salivary community, appears to be more prone to lifestyle habits such as smoking, agreeing with previous reports that the salivary community remains stable despite lifestyle-hygiene-related mediations such as flossing (David et al.,

2014, Shaw et al., 2017, Utter et al., 2016). This agrees with several prior reports of the stability and resilience of oral communities over time (Carpenter, 2020, Mason et al., 2018, Shaw et al., 2017, Kennedy et al., 2019, Abeles et al., 2016, Belstrom et al., 2016, Stahringer et al., 2012, Gomez et al., 2017, Premaraj et al., 2020a). Although host genetics help to shape microbial communities, previous reports of low variance between twins suggest that the shared early environment is the key determinant of the long-term composition (Shaw et al., 2017, Stahringer et al., 2012, Premaraj et al., 2020a). Longitudinal observations of twins revealed that the salivary microbiome has a stable core community at the genus level, and as twin lives diverge over time, environmental differences increase the diversity between the microbiomes of twins (Stahringer et al., 2012, Gomez et al., 2017). Furthermore, genetically unrelated people with a shared environment show similar environment-related effects on microbiome composition in the mouth as well as other communities (Shaw et al., 2017, Abeles et al., 2016, Gomez et al., 2017). Cohabitation appears to have a greater impact on the skin microbiome rather than gut and oral communities, persisting after the cohabitation is terminated (Shaw et al., 2017) (Utter et al., 2016, Stahringer et al., 2012, Blekhman et al., 2015a), an effect that is thought to persist for the long term despite leaving or changing household (Carpenter, 2020, Almeida-Santos et al., 2021, Mukherjee et al., 2021).

The importance of the OM should not be underestimated. As for the GM, there is a direct interaction between the microbiome and both oral and systemic health. Multiple oral inflammatory microbiome-associated conditions such as periodontitis and carries have strong epidemiological and mechanistic associations to other systemic and gastrointestinal diseases (Gomez et al., 2017, Byrd and Gulati, 2021). Further associations over the years have identified oral marker links to systemic complications, including cardiovascular, immune, metabolic, respiratory, osteopathic, obstetric, and perinatal complications (Byrd and Gulati, 2021, Gomez and Nelson, 2017, Li et al., 2000). In both healthy and inflammatory statuses, viable oral bacteria are often found to travel from the mouth to the gut and are capable of achieving successful colonisation (Prodan et al., 2019, Valdes et al., 2018, Ridlon et al., 2014). Schmidt et al. found that more than half of identified species often found residing in both mouth and gut exhibited signs of oral–gut transmission for all their study participants. Nearly one-third of these are taxa known to be highly dominant in oral communities (Prodan et al., 2019, Schmidt et al., 2019, Li et al., 2016). Interestingly, this is a one-way observation: although oral strains can travel to and colonise the gut, the opposite

is unlikely to occur (Prodan et al., 2019, Schmidt et al., 2019). Hence, as dental health research has been suggesting for years, oral microbial composition hinges on oral and dental health. In contrast to the prevailing GM, OM shows rising importance as an indicator of systemic health.

Although observational, we report numerous clear associations and correlations in our statistical model that demonstrate the crosstalk between the microbiome and the immune system. Microbial transmission across the gastrointestinal tract, direct microbial contact with tissue-resident innate cells, probable oral bacterial infection, circulating bacterial toxins, and molecular mimicry are all valid candidate pathways that may explain the observed relationship (Gomez and Nelson, 2017, Li et al., 2000). The ELM plays a crucial role in educating immune cells (immune tolerance) that are completely naïve at birth. As immune cells learn to recognise host cells, they are also programmed to recognise antigens from the developing beneficial endogenous microbiome (Zheng et al., 2020). Tissue-resident dendritic cells harvest microbial antigens from local microbial communities and present them to other immune cells (Zheng et al., 2020). In germ-free mice, the absence of a microbiome during the early-life period alters immune functions and induces structural defects in lymphoid tissues. In the presence of microbial communities, these tissue structures form normally. Despite many such observations, it is unclear how this acts mechanistically to alter the formation of epithelial barriers. Evidence from the gut suggests that bacteria can direct the glycosylation of luminally exposed surface proteins, a process whose outcome differs in germ-free mice (Round and Mazmanian, 2009, Bouskra et al., 2008, Bry et al., 1996). Initially, T_h17 cells are absent in germ-free mice and only appear upon microbial colonisation (Zheng et al., 2020, Round and Mazmanian, 2009).

It is now well-established that the relationship between stress and chronic disease starts in utero, as susceptibility and occurrence of disease can be predefined by maternal stress (Henriksen and Thuen, 2015). During this period, the naïve, uneducated, immune system develops (Fragkou et al., 2021). NK cells are part of the body's first line of immune defence, interacting with other immune cells as well as pathogens. In the majority of chronic diseases associated with the early-life environment, NK cells appear to either have an impaired function or an exaggerated cytotoxic activity (Ong et al., 2017, Yang et al., 2021). The most studied NK cell populations are the CD56brightCD16$^-$ and CD56dimCD16bright cells and the associated cytotoxic CD56dim and cytokine-producing CD56bright cells (Poli

et al., 2018). NK cell cytotoxicity is initiated by target cell contact and recognition, which leads to immune synapse formation, resulting in NK-cell-induced target-cell death. The proliferation and expansion of NK cells depend on $CD4^+$ T_h1 cells. Nevertheless, due to the bidirectional relationship between innate and adaptive immunity, NK cells impact $CD4^+$ and $CD8^+$ T cells through cytokine production (Abel et al., 2018). In the absence of short-chain fatty acids (SCFAs), metabolites produced from fibre fermentation by the local microbiome communities, certain CD4 T-cell subsets do not differentiate. Furthermore, naïve CD8 T cells do not differentiate into memory cells in germ-free mice (Zheng et al., 2020, Smith et al., 2013, Bachem et al., 2019). The activation of NK cells by pathogen-associated molecular patterns (PAMPs) may initiate an unwanted response in the microbiome and lead to a strong inflammatory response (Souza-Fonseca-Guimaraes et al., 2012). Similarly, pathogen-driven activation of NK cells can result in increased on-site cytotoxicity, which can also be harmful to local microbial communities. Correspondingly, microbiome members regulate homeostasis by inducing NK cell expansion and cytokine production or driving the proliferation of anti-inflammatory cytokine-producing NK cells, a common event observed with tissue-resident cells and microbiome crosstalk (Theresine et al., 2020). Furthermore, immunomodulatory properties of the bacterial community may drive antiviral defences regulating the outcome of viral infection (Przemska-Kosicka et al., 2018).

The OM is intimately linked to oral health. Poor oral health is often approached in an eco-social framework, as it is known to be associated with psychosocial adversity (Lee and Divaris, 2014). Both epigenetic and behavioural pathways were linked to poor oral health (Lee and Divaris, 2014). One of the most studied causal routes is diet. Affordability and access to a nourishing diet are strongly influenced by socio-economic status (Lee and Divaris, 2014), which in turn is linked to the composition of the OM. Detrimental shifts in the microbial composition associated with poor immune responses and mental health were documented for both hospitalised and long-term care home residents (Coman and Vodnar, 2020). The multidirectional interconnected relationship between the microbial composition, the host's immunological status, and the resulting life-long health trajectory is most probably highly dependent on constant exposure to particular irritants (Coman and Vodnar, 2020).

Our observation that psychosocial adversity is associated with changes in the OM opens many possibilities for future research. The collection of oral samples, primarily saliva, has been the sampling media of choice for psychobiology, lifestyle, and other social to clinical

research areas for many decades. Saliva has long been recognised as an accurate, noninvasive, and cost-effective diagnostic approach that can be tailored to personalised medicine strategies (Gomez and Nelson, 2017, Verma et al., 2018). Here, we opened up the possibility of using standard salivary swabs previously collected for microbiome studies. Such studies have the potential to provide a more holistic view of host–microbe interactions and the role of the microbiome in health, which is a potential that can now be applied in nearly all areas of psychobiology (and further afield). Our data also provide preliminary mechanistic insights and the perspectives for future detailed mechanistic studies. We know that early oral microbial colonisation is associated with IL-17-producing cells (Koren et al., 2021), and subsequent chronic oral disease is often initiated by T_h17 cells and IL-17 (Bellando-Randone et al., 2021, Abusleme and Moutsopoulos, 2017, Gaffen and Moutsopoulos, 2020). In our EpiPath cohort, there was a strong ELA-associated increase in immunosenescence-associated chronic inflammation, together with increased T_h17 cell numbers, although this narrowly missed significance ($p = 0.06$, (Elwenspoek et al., 2017a)). The ELA-associated immunophenotype is centred on immunosenescence (Elwenspoek et al., 2017a, Elwenspoek et al., 2017c). Here, we saw clear associations between *Selenomonas*, *Campylobacter*, and *Capnocytophaga* with T-cell immunosenescence, and together with the activated immature NK cell-associated *Alloprevotella*, these genera were all associated with periodontitis, gingivitis, and T2D. Diseases such as periodontitis and gingivitis have long been associated with changes in both the local and peripheral immune systems. This may be mediated by IL-17 from T_h17 cells, and it has been implicated in periodontitis-associated distal diseases in many disease contexts (Konkel et al., 2019, de Aquino et al., 2017). This is directly induced by microbial dysbiosis (Dutzan et al., 2018). Furthermore, direct microbial interaction with immune cells may underlie this, as loss of Toll-like receptor-2 (TLR2) in antigen-presenting cells reduces IL-17 secretion from T_h17 cells that dysregulate the host immune system in periodontitis (de Aquino et al., 2014). A similar direct link from the microbiome to the induction of a T_h17 cell response was previously reported for Streptococcus (Goncalves et al., 2016). As such, it is interesting to hypothesise that innate immune signalling from TLRs on immune cells within the oral cavity may directly mediate microbiome–immune interactions, acting locally and distally.

As with all investigations, our study is not without limitations. Due to the limited quantity of the biobanked samples, 16S sequencing was favoured over shotgun sequencing

to ensure good-quality data, leading to a limited taxonomic resolution in comparison to metagenomics studies. Future metagenomics studies are needed to refine the herein-presented association pattern, exploring potential differences within one genus. The EpiPath cohort consists of only 115 participants. This is a considerable number for a study on ELA, in which a full psychosocial stress test was performed, together with full immune and psychological profiling. However, this sample size is considered small for a microbiome studies, where statistical screening leads to multiple testing, reducing the statistical power for detecting individual associations. Similarly, the reported mediation analyses lack statistical power, and negative results should not be interpreted as the absence of effects. Similarly, as EpiPath is an adoption cohort, metadata such as the mode of birth, if they were ever collected, were never transferred to the adoptive parents. It is also possible that our data could be interpreted as the early inoculation with different microbiomes that simply persisted until 24 years later. The invasive nature of the ELA questions meant that compromise on microbiome-specific metadata, such as dietary habits and oral health status, was unavoidable if maximum participation in the study was to be ensured. Such information would have enhanced the mechanistic potential of our dataset. Knowledge of potential oral complications such as carries or periodontitis will be necessary in future studies to ensure that mechanistic pathways can be explored (Dimitrov and Hoeng, 2013). As the cohort consists of observational human data, causal interpretations of the reported associations should be treated with care. However, we demonstrated that 16S sequencing, despite its known limitations, provided clear insight into the long-term effect of ELA on the microbiome. Follow-up studies using shotgun metagenomics may refine the reported associations on the species and strain level.

2.4. Materials and Methods

Participants For this study, we used our previously reported EpiPath cohort of 115 adults aged 20 to 25 years (Hengesch et al., 2018, Elwenspoek et al., 2017a, Elwenspoek et al., 2017c, Elwenspoek et al., 2020). A total of 75 control participants were brought up by their biological parents and 40 participants were adopted in Luxembourg from institutions worldwide. The median age at adoption was 4.3 months (IQR 0–15 months) (Elwenspoek et al., 2017a). Basic immunoprofiling was available for all cohort members (Elwenspoek et al., 2017a, Elwenspoek et al., 2017c). Furthermore, detailed NK cell profiling was available for

76 participants (19 cases and 57 controls), and immunosenescence profiles were available for 79 participants (19 ELA and 60 controls) (Fernandes et al., 2021, Elwenspoek et al., 2017c). Biobanked oral swabs were available for 98 participants (33 ELA and 65 controls) and buccal swabs for all 115 participants (40 ELA and 75 controls). For one participant without immunosenescence profiling, the body mass index and sex were missing. This individual was excluded from statistical analyses, where the BMI and/or sex were used as covariates.

Oral samples Saliva samples were collected using Salimetrics Oral Swabs (Salimetrics, Cambridge, UK). Salivary cortisol levels have previously been reported from these samples (Hengesch et al., 2018, Elwenspoek et al., 2020). Buccal swabs were collected with Isoxelix Buccal Swabs (Isohelix, Harrietsham, U.K.). Microbial DNA was extracted using Qiagen DNA from a body fluids kit (Qiagen, Venlo, The Netherlands) according to the manufacturer's protocol. Samples were quantified with Qubit 1.2 (Invitrogen, Merelbeke, Belgium) and quality was assessed with a Nanodrop (Thermofisher, Merelbeke, Belgium). The V4 region of the 16S gene was amplified from bacterial DNA using 515F (Parada et al., 2016) and 806R (Apprill et al., 2015) forward and reverse primers (Eurogentec, Seraing, Belgium). The amplification reagents and library preparation were performed using a Quick-16S kit and its equivalent dual indexes (BaseClear, Leiden, The Netherlands) using the manufacturer's low microbial DNA concentration protocol. Libraries were quantified with Qubit, 1.2, 1.4 (Thermofisher, Merelbeke, Belgium); quality and size were assessed using a BioAnalyser (Agilent, Diegem, Belgium). Sequencing was performed on an Illumina MiSeq system with v2 sequencing chemistry and 500 bp paired-end reads, as well as 10% PHIX control according to the manufacturer's protocol.

Bioinformatic analyses Fastq files were processed, aligned, and classified using mothur 1.41v (Schloss et al., 2009). Alpha (inverse Simpson diversity index and Shannon evenness index) and beta diversity (Jaccard Index) were further calculated in the same pipeline. Sequences classification was aligned based on the Silva v138 database (Quast et al., 2013). Further integration of microbiome data into the immunophenotype and metadata as well as visualisations were performed with R.

Statistical analyses For descriptive statistics, nominal variables are described by proportions, while metric variables are described by means and standard deviations.

Evenness and Shannon entropy metrics were calculated for the OM as measures of alpha diversity and compared between ELA cases and controls with Wilcoxon rank sum tests. Additionally, diversity measures were compared across the OM using rank correlations. For investigating statistical associations between taxonomical units and immune-cell numbers, relative abundances for all genera were checked for outliers. Observations that were outliers both in immune-cell numbers and relative abundances (more than four standard deviations away the mean) were excluded from the analyses, when analysing genus–immune-cell associations. Only genera, or phyla, detected in more than 50% of all cases, were analysed. The microbial abundance data were analysed using fractional regressions (Baldini et al., 2020, Papke and Wooldridge, 1996). Fractional regressions are semiparametric methods not relying on distributional assumptions, and are specifically designed for the analyses of relative abundance data, making them suitable for the analysis of microbiome data, as different species abundances may not be sampled from the same class of distributions. Fractional regressions can be parametrised by odds ratios, allowing for easy interpretation of the regression coefficients in terms of the chance that a certain sequence read is assigned to a taxonomic unit (Baldini et al., 2020). All fractional regression models, if not specified otherwise, included age, BMI, and sex as covariates, and were performed separately for the OM communities. The basic covariates were included mainly to reduce residual variance and thereby increase statistical power to detect associations with the predictor of interests. Using fractional regressions, we screened the microbiome for associations with the study group variable, basic covariates (age, sex, body mass index (BMI), and smoking), antibody titres for Epstein–Barr virus (EBV), cytomegalovirus (CMV), and the herpes simplex virus (HSV), immunosenescence markers, as well as immune cell counts. All *p*-values are reported two-tailed. Statistical analyses were performed in STATA 16/MP (College Station, TX, USA), and correction for multiple testing was performed by applying the false discovery rate (FDR) (Benjamini, 2010). An FDR < 0.05 was considered to be significant. Summary statistics of the performed analyses are given in Supplementary Tables S1–S4.

2.5. Conclusions

Our data show a clear link between ELA and the OM that was visible 24 years later. The two oral communities investigated were clearly associated but distinct. We previously reported that ELA induced higher activation and senescence of the immune system. The taxonomic differences in the oral composition were not only associated with ELA but also with the immunosenescence of CD4 T cells, circulating numbers and activation status of NK cells, and anti-CMV titres. Although we do not yet have a detailed mechanistic explanation, our data suggest the presence of multiple links between ELA, immunosenescence, and cytotoxicity that persist through long-term changes in the microbiome.

2.6 Acknowledgments

The authors would like to thank Paul Wilmes and the members of the FNR-funded doctoral training unit “MicrOH” without whom this study would not have been possible. Furthermore, the authors would like to thank Lorieza Neuberger-Castillo for help with 16s-rRNA sequencing.

Supplementary Materials

The following are available online at https://www.mdpi.com/article/10.3390/ijms222312682/s1.

Chapter 3. (De)Bugging stress: does the microbiome determine our reaction to a psychosocial stressor?

My contribution to this Chapter: Conceptualisation; Data curation; Microbiome analysis; Data visualisation; Interpretation of results; Literature review; Writing of the article.

Abstract

Intense psychophysiological stress in early in life has a detrimental effect in health-disease balance in later life. At the same time, despite its sensitivity to stress, the developing microbiome also contributes on the long-term health trajectory. Following stress experience, activation of HPA axis regulates “fight or flight” response with the release of glucose and cortisol following a poorly understood complex mechanism. Our study investigates the interaction between the oral microbiome and the stress response. We used a cohort of 115 adults, mean age 24, who either experienced institutionalisation and adoption (n=40) or were non-adopted controls (n=75) of which glucose and cortisol measurements were taken following an extended socially evaluated cold pressor test. We then examined the oral microbiome of the cohort via 16S V4 amplicon sequencing on microbial DNA from saliva and buccal samples. Taxonomic analyses were performed with mothur. Statistical analysis was performed on Stata. We identified 12 taxa that exhibited an interaction with host`s cortisol-glucose response to stress. Our data expose an interaction of the oral microbiome with host`s stress response. In particular the identified taxa defined when glucose and cortisol would peak and determined their kinetics following seCPT exposure.

Keywords: early-life adversity, early experience, microbiome, bacterial community, oral microbiome, developmental origins of health and disease, stress, cortisol, glucose host-microbe interactions

3.1. Introduction

The physiological stress response is the body's natural reaction to external stressors that causes physical, psychological or emotional strain. While the physiological response to the stressor is part of every day life, there are periods of life during which we are particularly affected. Stressful events occurring very early in life are particularly harmful, and are intimately linked to health-disease balance in later life (Grova et al., 2019, Turner, 2018a). In the mid-1980s Barker and Osmond introduced the developmental origins of health and disease (DOHaD), where the environment in the first 1000 days was hypothesised to shapes health and disease profiles lifelong (Barker and Osmond, 1986). Although Barker and Osmond were initially interest in foetal nutrition, this has now expanded to cover all negative experiences in this 1000-day period, and has led to interest in early-life adversity (ELA). ELA is a rather diffuse concept, covering many different forms of potentially adverse environmental exposure within the first 1000 days. Many studies have subsequently investigated the molecular mechanisms linking ELA to psychobiological, behavioural, immunological and disease phenotypes (Seal et al., 2022, Elwenspoek et al., 2017b, Elwenspoek et al., 2020, Hengesch et al., 2018, Wampach et al., 2018, Shao et al., 2019, Reyman et al., 2019, Sarkar et al., 2021, Yang et al., 2016, Moore and Townsend, 2019, Backhed et al., 2015).

Upon exposure to a stressor, the autonomic nervous system and the hypothalamus pituitary adrenal (HPA) axis activates and coordinates the "fight or flight" response via the release of catecholamines and glucocorticoids (Seal et al., 2022, Seal and Turner, 2021, Bowland and Weyrich, 2022). In parallel glucose is produced and released (Seal and Turner, 2021). The current dogma is that this is a glucocorticoid-mediated process. Exposure to ELA has lifelong effects on HPA axis regulation and glucocorticoid levels, consequently dysregulating glucose release and metabolism dynamics (Seal et al., 2022, Hengesch et al., 2018). However, how this process occurs is not yet well studied.

The microbiome, seeded at birth, is also known to have a pivotal role on the establishment of an individual's long-term health trajectory. Furthermore, both the oral and gastro-intestinal microbiomes are shaped by ELA (Charalambous et al., 2021, Reid et al., 2021, Wampach et al., 2018, Shao et al., 2019, Reyman et al., 2019, Sarkar et al., 2021, Yang et al., 2016, Moore and Townsend, 2019, Backhed et al., 2015). There has been a recent increase in interest in the oral microbiome (OM). After exposure to ELA dysbiosis of the

OM, with increased abundance of pathogenic taxa, leads to poor oral health. Poor oral health is part of the pathophysiological presentation of many ELA-associated diseases, including cardiometabolic, mental, autoimmune and allergic diseases. Furthermore, the paradigm of gut – brain – axis and oral – brain – axis is now more widely studied, strengthening the evidence of a constant communication between the gut and oral microbial communities with their hosts (Paudel et al., 2022, Bowland and Weyrich, 2022). Interestingly, not only is the OM sensitive to ELA, but it is also sensitive to both cortisol and glucose that may be dysregulated by ELA (Bowland and Weyrich, 2022, Carpenter, 2020, Kennedy et al., 2019). This raises the interesting hypothesis that dysbiosis in the microbiome interacts with the stress response. Moreover, this may represent a mechanism by which ELA alters the stress response

Here, we use our EpiPath cohort of ELA-exposed individuals and controls, in which both the acute stress response and the OM have been shown to be reshaped by early life psychosocial stress exposure (Hengesch et al., 2018, Seal et al., 2022, Charalambous et al., 2021) to study the role of the OM in host`s stress reaction.

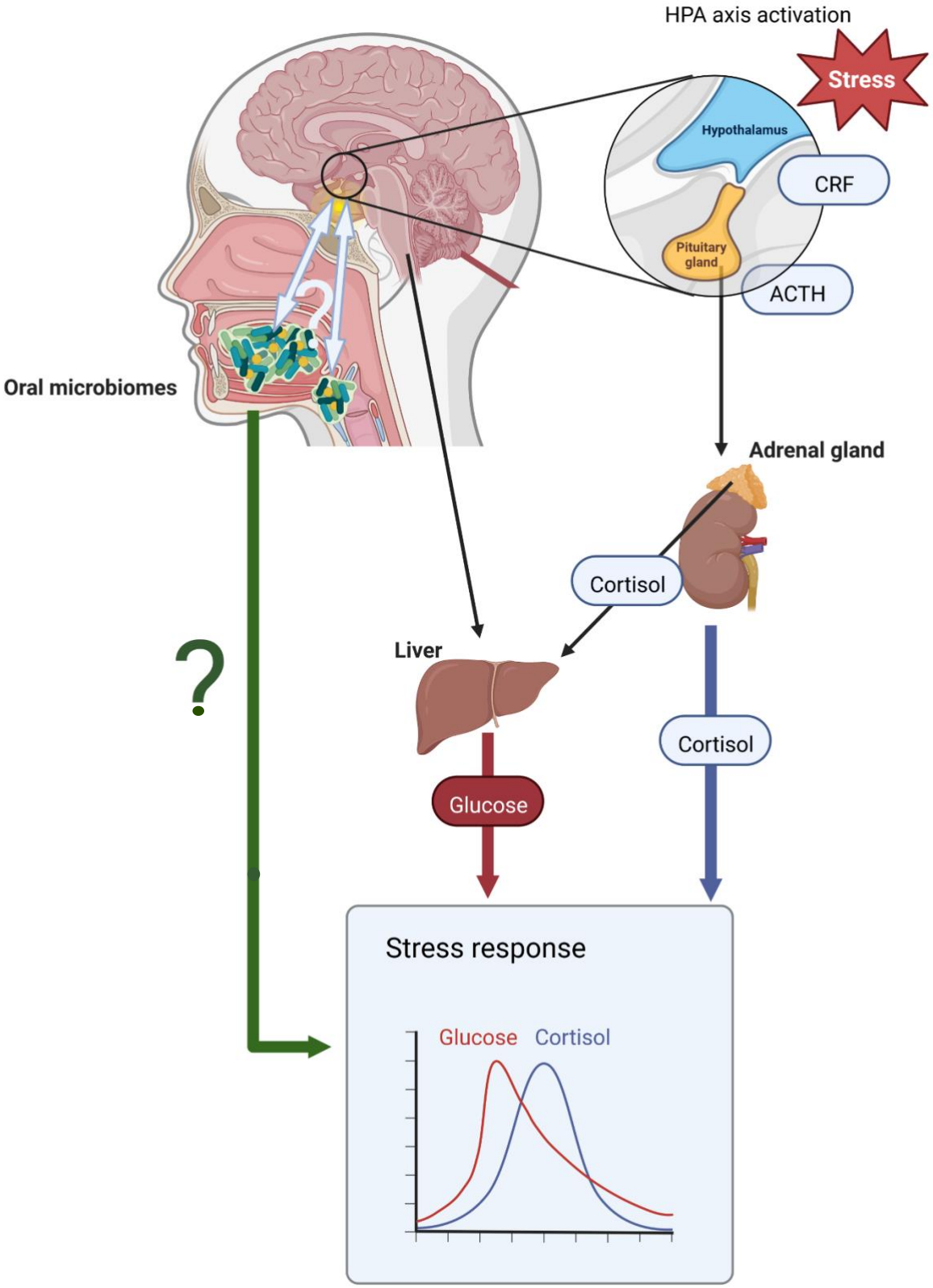


Figure 3.1. Summary outline

3.2. Materials and Methods

Participants and bacterial abundance data

In this study we used previously published buccal and salivary microbiome abundance data from sequencing of the V4 region of the 16S gene in our EpiPath cohort (Charalambous et al., 2021). EpiPath is a cohort of 43 post-institutionalisation adults and 73 control participants were brought up by their biological (Hengesch et al., 2018, Elwenspoek et al., 2017b, Elwenspoek et al., 2017a, Elwenspoek et al., 2017c). Microbial abundance data were from buccal swabs and salimetrics oral swabs taken upon arrival at the clinical centre. A subset of the EpiPath cohort (22 control and 22 ELA) underwent a socially evaluated cold pressor test (seCPT; (Hengesch et al., 2018)), and were used in this study. Median age at adoption was 4.3 months (IQR 0-15 months) (Elwenspoek et al., 2017a). EpiPath was approved by the Luxembourg National Research Ethics Committee (CNER, No 201303/10 v1.4) as well as the University of Luxembourg Ethics Review Panel (ERP, No 13-002). All participants provided written informed consent in compliance with the Declaration of Helsinki. All study participants received a small financial compensation for their time and inconvenience.

Stress test: As previously reported, a subset of the cohort underwent an extended socially evaluated cold pressor test during which they were asked to place both feet into 2-3°C water for 3mins while performing a mental arithmetic task (Hengesch et al., 2018, Larra et al., 2014). Blood and saliva were collected using EDTA coated tubes and Salimetrics Oral Swabs respectively at -120 min, -5 min, +3 min (stress cessation), and then at 15, 30, 60, 120 and 180 minutes relative to T=0 when the participant placed their feet in the water. EDTA tubes were centrifuged at 4 C for 15 minutes and plasma collected. Samples were stored at -80 C prior to utilisation.

Cortisol and glucose Measurements

Salivary cortisol from 70 participants was measured as described by Hengesch et al (Hengesch et al., 2018) using the Salimetrics Salivary Cortisol ELISA kit (CV: 7% intra-assay, 11% inter-assay, Salimetrics, Cambridgeshire, UK). Glucose was measured from thawed, plasma samples of 42 participants. These, were briefly vortexed and placed on a fresh Accu-Chek strip (Accu-Chek, Roche) to quantify the plasma glucose concentration as described by Seal et al (Seal et al., 2022).

Statistical analyses

For descriptive statistics, metric variables were expressed as means +/- standard deviations, categorical variables were expressed via proportions. For interaction analyses of the response to the CP with the oral microbiome, we utilised two types of analyses. First, for genera being present in 25-75% of all samples, we dichotomized the abundance of those genera (genus present vs. genus absent). Then, we conducted for each of these genera a linear mixed model with the plasma glucose, respectively saliva cortisol concentration, being the response variable. These mixed linear regression models included the age, sex, body mass index, and the time of measurement as fixed effect covariates and the individual as random effect variable. For plasma glucose, measurements at six time points were available for each individual, while for saliva cortisol measurements at 16 time points could be analysed. The time variable was treated in minutes with the time point of the CP test being set to zero. Importantly, the time point of measurement was treated as a categorical variable to allow for the expected non-linear response over time to the CP test. We then introduced interaction terms between the categorical time point variable and the dichotomized species presence and tested the model including the interaction terms against a model including all named covariates plus the dichotomized genus abundance through likelihood ratio tests. The likelihood ratio tests effectively test, whether the saliva cortisol, respectively plasma glucose, response is the same for individuals having a certain genus vs. not having a certain genus in their oral microbiome. This string of mixed linear regression models was performed for both the oral and the buccal microbiome. Second for genera being present in more than 50% of the samples, we performed analogous interaction analyses, utilising however the metric abundance variable instead of the dichotomized variable. Once again, significance was determined by likelihood ratio test of the model including covariates, main effects and time-point genus abundance interactions terms vs. the model only including covariates and main effects. All reported p-values are two-tailed. Statistical analyses was performed with STATA 16/MP and the mixed models were performed using the “xtreg” command with the option “mle” to specify maximum likelihood estimation. We corrected for multiple testing using the false discovery rate (FDR) and an FDR<0.05 was considered to be significant.

3.3. Results

As previously reported, glucose and cortisol levels are raised by the seCPT in the EpiPath cohort. The exposure to institutionalisation-adoption made subtle but statistically significant changes to the kinetic profile of the stress induced release of cortisol and glucose. Briefly, we saw a rise in glucose levels from 101.8 +/- 12.6 mg/dL to 136 +/- 21.7 mg/dL and cortisol rose from 0.27 +/- 0.14 µg/dL to 0.55 +/- 0.29 µg/dL.

Table 3.1. Summary statistics

Taxa	Buccal microbiome	Salivary microbiome	Interaction analysis
Absconditabacteriales	cortisol	-	presence/absence
	glucose	-	presence/absence
Acinetobacter	cortisol	-	presence/absence
Clostridia UCG14	cortisol	-	presence/absence
Campylobacter	-	cortisol	presence/absence
Cardiobacterium	-	cortisol	presence/absence
Oxalobacteraceae	glucose	-	presence/absence
Sphingomonas	glucose	-	presence/absence
	glucose	-	abundance
Bradyrhizobium	glucose	-	abundance
Comamonadaceae	glucose	-	abundance
Flavobacterium	glucose	-	abundance
Methylobacterium - Methylobrubrum	glucose	-	abundance
Paucibacter	glucose	-	abundance

Here, we took the two arms of the EpiPath cohort together, using the exposure to ELA as a source or variance in metabolic and hormonal responses to stress coupled with differences in the microbiome. In the combined arms of the cohort, both glucose and cortisol showed a clear stress-induced rise that eventually falls to backgrounds. Analysing the cortisol and glucose time series via linear mixed effect regression models, we identified a total of 12 taxa, 10 that were present in the buccal and 2 in the salivary microbiome prior to the stress test that subsequently interacted with either the cortisol or the glucose response to the seCPT. Significant interactions are summarised in Table 1.

3.3.1 Cortisol response depends on the oral taxonomic profile

Having identified taxa that associated with the metabolic and hormonal response to stress, community relative abundances were dichotomized coding for presence or absence of *genera*, enabling an interaction analyses between species presence (binary: absent vs. present) and cortisol response to the cold pressor test based on linear mixed regressions adjusted for age, sex, BMI and ELA. Our results revealed 3 taxa from the buccal community interacting with the salivary cortisol response to stress in the buccal community. *Absconditabacteriales* and *Clostridia UGC14* presence had no significant effect on the baseline levels of cortisol. However, their presence was associated with prolonged clearance of cortisol following stress (Fig2 A – FDR=0,726059508, C – FDR=8,05E+00, Fig B and D - FDR<0.05). In contrast, *Acinetobacter* presence, while also having no significant effect on the baseline levels of cortisol, was associated with accelerated clearance of cortisol (Fig2 E – FDR=1,41E+01, Fig2 F - FDR<0.05). In addition, analyses of salivary communities showed 2 *genera* interacting with cortisol levels in saliva after the CP test, while none of the genera had an effect on baseline cortisol levels after correction for multiple testing. *Campylobacter*`s presence was associated with a lower cortisol reaction to CP in general. (Fig2 G – FDR=1,12E+00, Fig2 H - FDR<0.05). *Cardiobacterium* presence, on the other hand, was associated with a a higher cortisol peak but faster clearance (Fig2 J – FDR=1,22E+01, Fig2 I - FDR<0.05). In conclusion, community composition both in the saliva as in the buccal microbiome interacted with the measured cortisol response to CP.

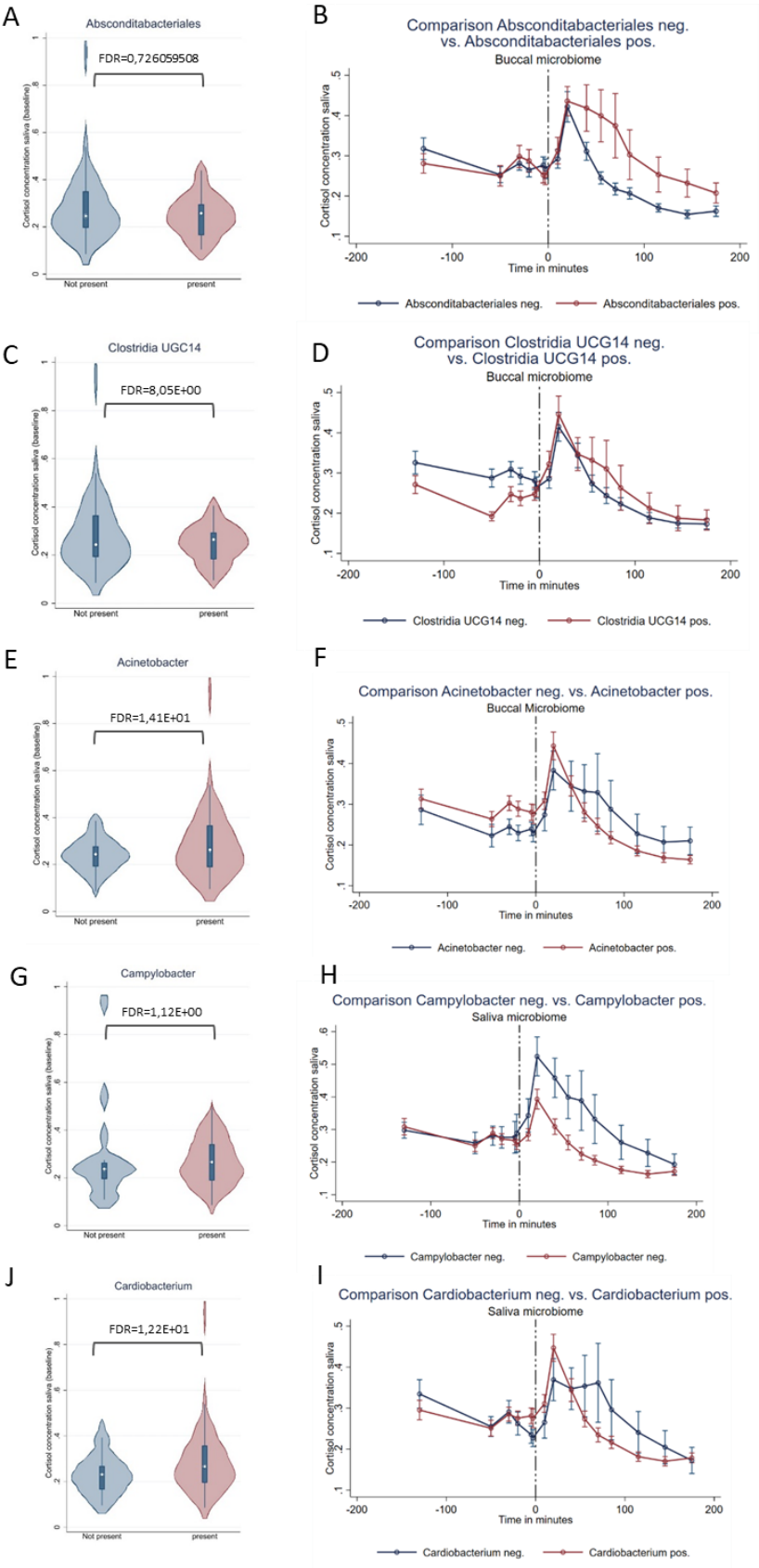


Figure 3.2. Presence of oral taxa defines stress-induced cortisol kinetics.

3.3.2 Glucose response depends on the oral taxonomic profile

Next, we explored the relation between glucose response and oral microbiome relation. To this end, we conducted linear mixed regression modelling analyses in an analogous way as described above, utilizing however the plasma glucose levels as response

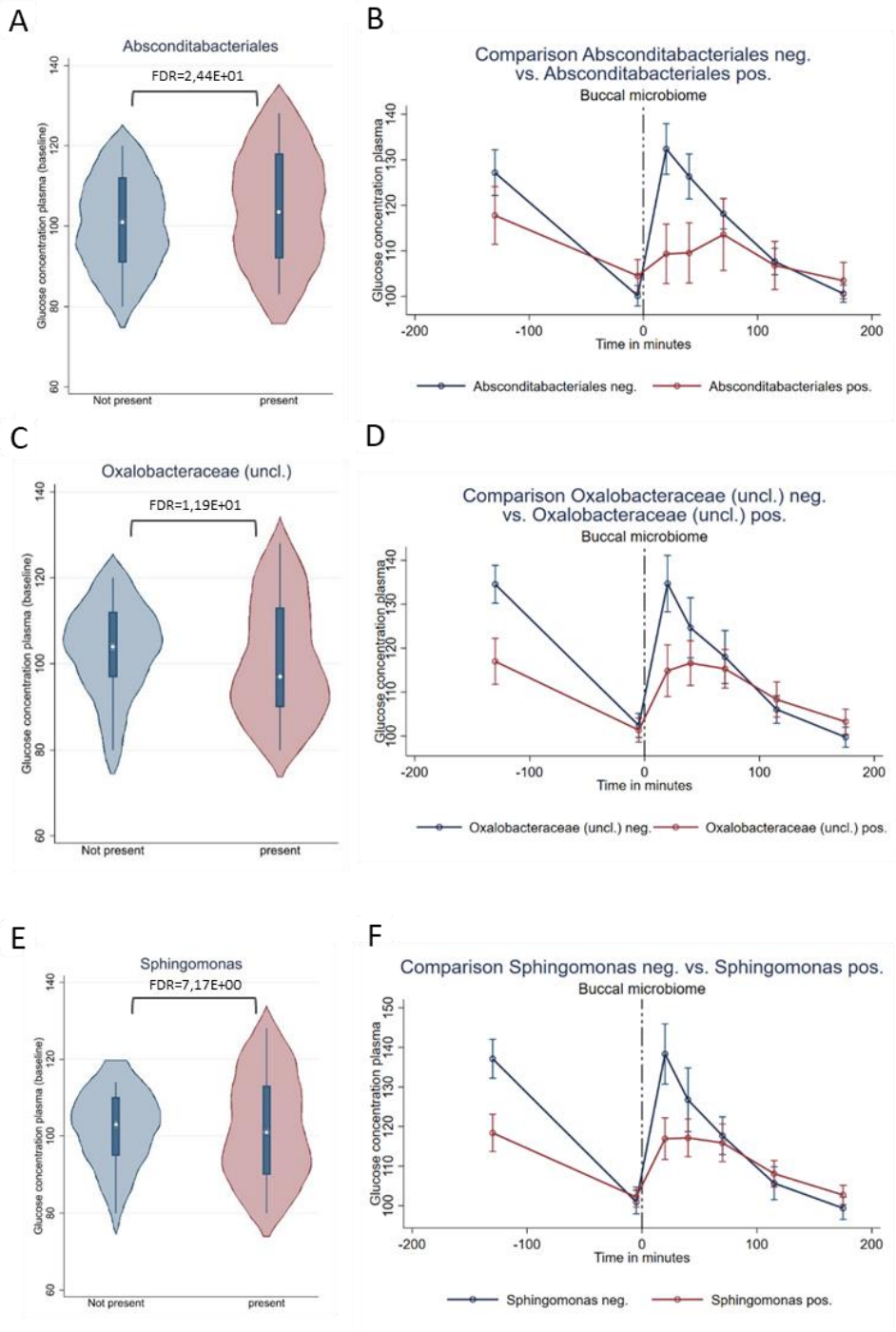


Figure 3.3. Presence of buccal taxa defines stress-induced glucose kinetics.

variable instead of the saliva cortisol concentrations. While the salivary bacteriome showed no interactions, three taxa from the buccal community appeared to interact with glucose stress response despite showing no significant association with the baseline plasma glucose levels 5min before stress test. *Absconditabacteriales* presence was associated with muted plasma glucose response, while participants with *Absconditabacteriales* in the buccal microbiome showed a strong glucose response (Fig3 A – FDR=2,44E+01, Fig3 B - FDR<0.05). Moreover, *Oxalobacteraceae* and *Sphingomonas* was associated with a reduced glucose response (Fig3 C–FDR=1,19E+01, E–FDR=7,17E+00, Fig3 D, F - FDR<0.05). Notably, both species co-occurred with each other in the analyzed buccal microbiomes, showing therefore parallel association patterns with plasma glucose levels (Fig3 C, E.)

3.3.3 Glucose clearance associates with a higher taxonomic abundance

While in the analyses above, we worked with dichotomized genus variables, in the next string of linear mixed regression models, we utilised dimensional interaction terms between abundances and plasma glucose, respectively saliva cortisol, levels. Neither the buccal, nor the saliva microbiome showed any significant interactions on the abundance level after correction for multiple testing with the saliva cortisol levels. However, for six genera in the buccal bacteriome, the abundance was associated with clearly altered the glucose response (Figure 4). For visualization of the dimensional interaction terms, abundances were stratified in according to tertiles. *Bradyrhizobium*`s presence in high abundance (>66th percentile) was associated with delayed and lower peak, while showing longer clearance in tendency (Fig4 A FDR<0.05). Abundances of *Commamonadaceae*, *Flavobacterium* and *Sphingomonas* showed a similar interaction where lower abundances 0-33th percentile and 33th - 66th percentile had a similar response, whereas individuals with abundance of these taxa >66th percentile exhbited a muted glucose response with a less clear peak. (Fig4 B-D, F FDR<0.05). Furthermore, 0-33th percentile and 33th - 66th percentile abundances of *Methylobacterium-Methylorubrum* showed a very similar response while the individuals with abundance higher than 66th percentile demonstrated a lesser glucose response with longer clearance (Fig4 E FDR<0.05). Last, *Paucibacter*`s abundance showed a different interaction with the glucose stress response. Individuals with less than 33th percentile of *Paucibacter* showed

a clear glucose response to CP, while individuals with abundances in the two lower tertiles had a lower glucose response (Fig4 F FDR<0.05).

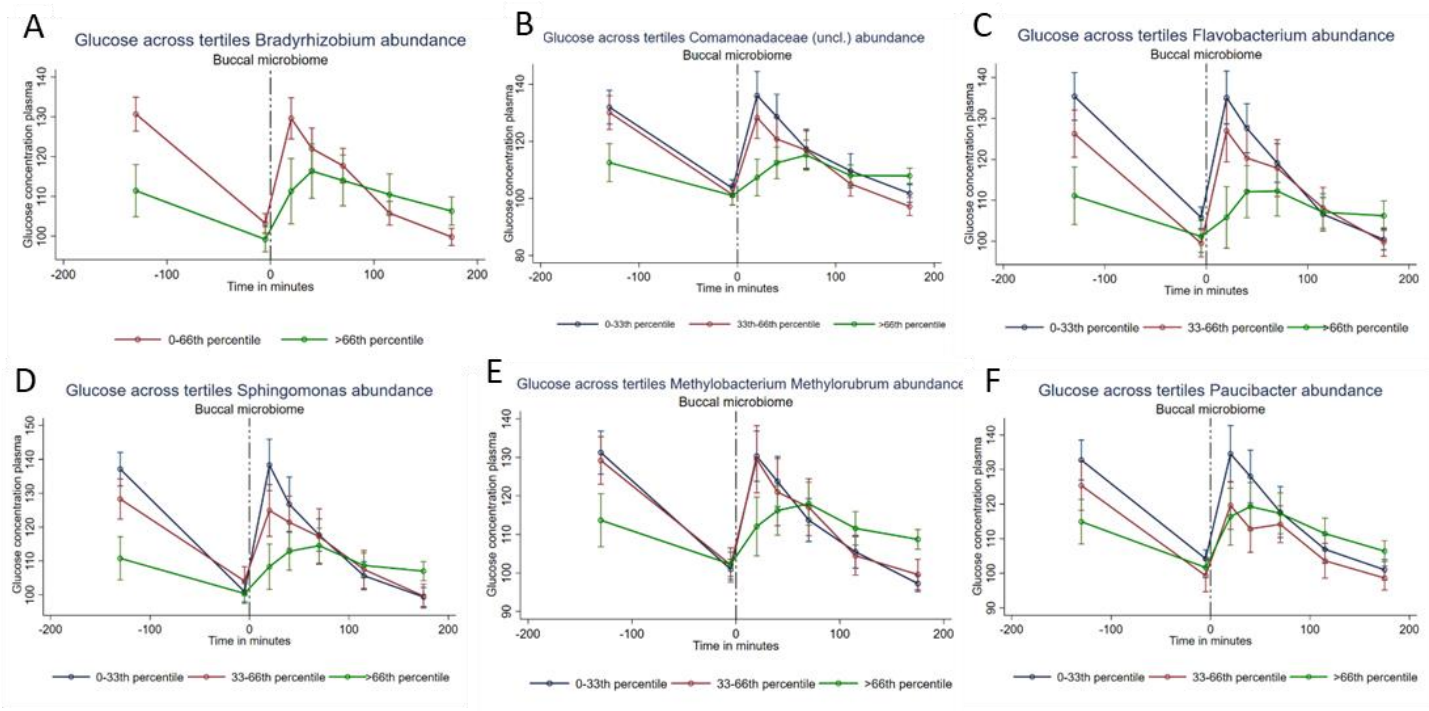


Figure 3.4. Relative abundance of buccal taxa defines stress-induced glucose kinetics.

3.4. Discussion

In this study we showed that there was a clear link between the composition of the oral microbiome and both the hormonal and metabolic response to a laboratory psychosocial stressor. Our EpiPath cohort that had been exposed to early-life adversity 20 years earlier had both a reshaped stress responses and microbiota, providing variance in both to enable us to identify interactions between bacterial taxa and the stress response. Within this paradigm, the stress-induced cortisol response was associated with 5 bacterial taxa originating from either the buccal or salivary microbiomes. Furthermore, 8 taxa from buccal microbiome when present, or present in increased abundance interacted with the stress induced glucose response, determining the kinetics of glucose following seCPT exposure. Overall our data demonstrate that the oral microbiome interacts with HPA axis activation.

Many studies have linked ELA to a dysregulated HPA axis stress response (Cornejo Ulloa et al., 2019, Steele et al., 2015, Boyce et al., 2010). Concurrently, numerous studies investigating gut-brain axis and/or oral-brain axis have reported that microbiota respond to

host hormones resulting to change in bacterial gene expression (Kim et al., 2022, Bowland and Weyrich, 2022, Jentsch et al., 2013, Garcia-Pena et al., 2017, Selway et al., 2020, Cutler et al., 2019, Muras et al., 2022). In particular, evidence from the oral microbiome suggest that exposure to high cortisol levels results to upregulation of virulence factors (lipopolysaccharide, fimbriae or gingipaıns) in periodontitis associated taxa, *Fusobacterium* and *Poprhymonas* resulting to a global shift of the composition towards a pathogenic type (Kim et al., 2022, Bowland and Weyrich, 2022, Akcali et al., 2013, Roberts et al., 2002, Jentsch et al., 2013). This ability of the OM to react to the host`s hormonal response is part due to resilience mechanisms determining the community composition and driving homeostasis (Cornejo Ulloa et al., 2019, Alcon-Giner et al., 2020). During the HPA activation and stress response, in addition to cortisol, catecholamines are also released. In vitro studies have shown that these can promote or inhibit the growth of OM taxa known to be associated with periodontitis and evidently lead to stress induced taxonomic shifts in the OM composition (Cornejo Ulloa et al., 2019, Roberts et al., 2002, Jentsch et al., 2013, Gupta, 2014). In a similar manner, our data suggest a clear interaction between the host's stress hormones and the composition of the microbiome. However, the directionality of the interaction remains unclear. It is possible that the host's stress response is modified by the microbiome. On the other hand, however, 24 years after institutionalisation, it is more probable that, as in vitro, the microbiome has established a new homeostasis based on the ELA-specific stress and metabolic profile of the individual, with stress hormones and glucose promoting or inhibiting growth of specific taxa.

Glucose can be a substrate for certain taxa that is used in pentose phosphate pathways whilst certain taxa such as *Sphingomonas* can produce glucose through other metabolic pathways (Vartak et al., 1995, Martins and Sa-Correia, 1991, Gorke and Stulke, 2008, Gatta et al., 2022). Additionally, *in vitro* studies suggest that glucose concentration affects bacterial mobility and more precisely the swimming – swarming behaviour (De et al., 2021, Armitage et al., 1976, Lai et al., 1997, Mattingly et al., 2018, Kearns, 2010). Swarming is often observed in stress or disease state (De et al., 2021). This is believed to occur through a glucose-dependent quorum sensing mechanism (Gatta et al., 2022, Jahid et al., 2013, Mattingly et al., 2018). This process requires different carbon sources for carbon catabolite repression (CCR) which is broadly believed to be glucose (Gatta et al., 2022, Yang and Lan, 2016). Additional in vitro data suggests that glucose is the most accessible and preferred

carbon source, driving faster bacterial growth (Gorke and Stulke, 2008, Jahid et al., 2013). CCR dependent quorum sensing contributes to an increase in virulence proteins (Gatta et al., 2022, Lee and Zhang, 2015, Rumbaugh et al., 1999, Yang and Lan, 2016).

In this complex system where ELA and stressful events can lead to dysbiosis and a ruffled balance of health-disease the light falls in the microbiome in order to understand underlying mechanisms of action. The microbiome and more importantly the oral communities have been shown to be involved in the modulation of neurological process, to shape behaviour and even cognition by interacting with the neuroendocrine system (Bowland and Weyrich, 2022). This modulation may, in part, be due to metabolites or other small molecules secreted by the microbiome and absorbed by the host. On the other hand, as saliva contains molecules with antimicrobial properties, the OM has also evolved to recognize and respond to such signals (Carpenter, 2020, Kennedy et al., 2019, Almeida-Santos et al., 2021, Mukherjee et al., 2021, Jakubovics, 2015). This re-enforces the idea that there is an equilibrium or homeostasis between the hosts stress response and the OM composition.

The OM possess a set of sociomicrobiological skills, scientifically known as quorum sensing (QS), a form of intra- and inter- species communication that allows the OM to sense and modulate the host host (West et al., 2006, Bowland and Weyrich, 2022, Plancak et al., 2015). QS mechanisms are the basis of how bacteria react and regulate stress that can ensure survival and homeostasis of a functional bacterial community (Bowland and Weyrich, 2022, Garcia-Contreras et al., 2015). Both cortisol and glucose are known to interact with QS whilst individual taxa are able to counteract by releasing particular autoinducer peptides, which often enhances their virulence and growth-speed (Plancak et al., 2015, Verbeke et al., 2022, Frias et al., 2001). Furthermore, some microbial metabolites can neutralise the action of the QS peptides aiming to promote homeostasis within the community, such metabolites are D-Galactose and D-arabinose (Wright and Ramachandra, 2022). These properties of the OM have ensured long-term stability, resilience and robustness for its communities (David et al., 2014, Shaw et al., 2017). Furthermore, it is the key on how oral taxa can drive oral inflammation and impact systemic health later on (Coman and Vodnar, 2020). Overall these pre-existing evidence explain the observations of our study and strengthen our hypothesis that the OM interferes with HPA axis activationa and glucose-cortisol stress response. Further mechanistic-focus studies are essential to explore exactly how each of these QS

mechanisms and bacterial metabolic properties relate to this OM-HPA interaction. As such, it would be interesting in future studies to measure salivary QS molecules to examine their role in determining the microbiome stress interaction.

Our observation that host`s stress response can be altered based on the composition of the oral microbiome brings opportunities for future research. Collection of oral swabs is widely performed in many research fields including psychobiology, lifestyle and other social to clinical research areas. Such samples are consisting a non-invasive, cost-effective and accurate diagnostic approach that is optimisable into personalized medicine strategies (Gomez and Nelson, 2017, Verma et al., 2018). The plausible use of such samples can enlighten investigations on the interaction of host-microbe and the role of the microbiome in oral and systemic health.

Our study, like all others, has limitations. The greatest limitation is that since it is a purely observational study we were not able to identify the direction of the interaction between the stress reaction and changes in the microbiome. To do so requires further detailed mechanistic studies. Additionally, the glucose and cortisol measurements from the EpiPath cohort were only measured for a smaller set of participants (cortisol n=70, glucose n=42). Despite the fact that this is a considerable number for a study on ELA, this sample size is considered small for a microbiome study. In addition, due to the original scientific question that the EpiPath was conducted for, microbiome-specific metadata such as, diet habits and oral health status were not collected. Knowing that some of the taxa we identified to interact with the stress response including *Sphingomonas* (van Winkelhoff et al., 2016, Genco et al., 2019), *Campylobacter* (Lenartova et al., 2021, Macuch and Tanner, 2000) and *Acinetobacter* (Vijayashree Priyadharsini et al., 2018, Perez-Chaparro et al., 2014) are taxa known to be associated with periodontitis, therefore such information on the participants would have strengthen the mechanistic potential of our dataset (Dimitrov and Hoeng, 2013). Nevertheless, we have, for the first time, clearly shown from this observational study that there is a clear interaction between taxa from the oral microbiome and the host`s stress response. In the case of our EpiPath ELA cohort, it is most probable that a dysregulated endocrine stress reaction is able to cause dysbiosis of the OM. This interaction between the two systems may play a role in re-establishing homeostasis of the OM following a stress trigger.

3.5. Conclusion

Our data show a clear alteration of the host's stress response as evidenced through the analyses of repeated glucose and cortisol measurements in relation to the taxonomic composition of the oral microbiome. We have, for the first time, clearly shown from this observational study that there is a clear interaction between taxa from the oral microbiome and the host's stress response. In the case of our EpiPath ELA cohort, it is most probable that a dysregulated endocrine stress reaction is able to cause dysbiosis of the OM. This interaction between the two systems may play a role in re-establishing homeostasis of the OM following a stress trigger.

Chapter 4. Early life adversity defines the metabolic capacity of the oral microbiome and associates with immune changes 20 years later.

My contribution to this Chapter: Conceptualisation; Literature review; Metabolic reconstructions, experimentation and analysis; Final data integration; statistical analysis; Data visualisation; Interpretation of results, manuscript writing.

Abstract

Early Life Adversity (ELA) is defined by exposure to a highly adverse, stressful, toxic, poor environment and difficult conditions during the first 1000 days of life. During this time the Oral Microbiome (OM) is seeded by birth route and its composition evolves over the same period. Exposure to ELA has been linked to a complex psychopathophysiological profile characterised by a pro-inflammatory and immunosenescent signatures as well as an ELA OM taxonomic profile in early adulthood. In an institutionalisation-adoption model of ELA we aim to investigate what is the lifelong metabolic impact on the OM. Previously generated 16S V4 amplicon sequencing data and taxonomic composition from salivary and buccal microbial of the EpiPath cohort were used to perform metabolic reconstructions and predict the metabolic profile of the participants. EpiPath is a cohort of 115 adults, mean age 24, who either experienced institutionalisation and adoption (n=40) or were non-adopted controls (n=75). Metabolic modelling of the oral microbiome accomplished with constraint-based reconstruction and analysis (COBRA) in Matlab. Data analysis, integration and visualisation were performed with R. We identified multiple significant metabolic associations dependent on the exposure to ELA, differences driven by biological sex, smoking, usage of contraception, exposure to early life *herpesviridiae* infection and multiple immunophenotypic markers of NK and T cells circulating numbers and activation status (BH<0.05). Our data provide the first evidence of an ELA imprint on the metabolome of both the salivary and buccal oral microbiomes that remain identifiable in early adulthood. Our results compose the first mechanistic indication of how the interaction between ELA-OM results to phenotypic changes.

Keywords: early-life adversity, oral microbiome, oral metabolome, microbial metabolites, immune system, host-microbe interactions, developmental origins of health and disease,

4.1. Introduction

Exposure to an adverse, stressful, toxic, poor environment and difficult conditions very early in life is what defines early life adversity (ELA). The result of ELA is a lifelong disparity of health and disease (Grova et al., 2019, Turner, 2018a). The developmental origins of health and disease (DOHaD) theory was introduced in the mid-1980s by David Barker, who demonstrated that the environment in the first 1000 days of life determined an individual's lifelong cardiometabolic health (Barker and Osmond, 1986). Initially Barker focussed on foetal nutrition, but this has been expanded to all negative experiences, during this period. Following on from the work of Barker, a diverse series of studies have identified how ELA induces diverse lifelong differences, including psychobiological, behavioural, immunological and microbial, as well as disease phenotypes (Seal et al., 2022, Charalambous et al., 2021, Elwenspoek et al., 2017a, Elwenspoek et al., 2017c, Elwenspoek et al., 2020, Hengesch et al., 2018, Wampach et al., 2018, Shao et al., 2019, Reyman et al., 2019, Sarkar et al., 2021, Yang et al., 2016, Moore and Townsend, 2019, Backhed et al., 2015).

Initial work to identify the mechanism by which ELA has lifelong effect was centred around the immune system. Focus has more recently moved to the microbiome. Within this context, we recently investigated long-term changes in the oral microbiome (OM). The OM consists of several specific smaller communities including salivary and buccal microbiomes (Carpenter, 2020, Boustedt et al., 2015, Chu et al., 2017, Mason et al., 2018, Mark Welch et al., 2019, David et al., 2014, Shaw et al., 2017). Exposure to ELA-induced taxonomic changes in the OM that associated with ELA and immune system markers 24 years after exposure (Charalambous et al., 2021, Elwenspoek et al., 2017a, Elwenspoek et al., 2017c, Elwenspoek et al., 2020). In the institutionalisation-adoption model of ELA there is now a clear link between increased senescence in T-cells that is associated with a specific taxonomic profile in both oral and gut microbiomes. Additional associations on the same models linked the microbiome and the immune system including the senescent CD57 marker and other immune cell activation markers (Elwenspoek et al., 2017c, Holland et al., 2020, Reid et al., 2019, Reid et al., 2021, Charalambous et al., 2021). Other studies on early life development showed that the early oral microbial colonisation associates to IL-17-producing cells (Koren et al., 2021) while investigations on host-microbe interactions in relation to

chronic oral disease showed the microbiome to drive a Th17 cells and IL-17 shifted immune response (Bellando-Randone et al., 2021, Abusleme and Moutsopoulos, 2017, Gaffen and Moutsopoulos, 2020).

Furthermore, in line with the 3-hit hypothesis of ELA in which early life environment impacts the health trajectory, the microbiome included as part of the exposome, makes up the "second-hit' contributing to the programming of an emerging phenotype (Daskalakis et al., 2013). During this period the early life microbiome (ELM) is seeded and starts developing. This process is heavily dependent on this exposome, like many of the host`s other biological systems. Interestingly, salivary antimicrobial IgA antibodies can be detected from the 2nd week of life upon exposure to pathogens (Brandtzaeg, 2013). The presence of such molecules at this early stage subsequently interferes with the developing microbiome as the first evidence of host-microbe communication (Brandtzaeg, 2013).

Microbial metabolites are biochemical compounds produced when a microbe metabolizes nutrients for maintaining its energy levels and survival (Takahashi, 2015, Krautkramer et al., 2021, Horak et al., 2019). The metabolite pool of each individual microbe or the metabolite pool of a microbial community is what has been defined as the "metabolome" (Horak et al., 2019, Tang, 2011, Aldridge and Rhee, 2014). Hence, the metabolome is categorised into primary and secondary metabolites and consists of different classes of compounds including carbohydrates, sugars, proteins, peptides, amino acids, short-chain fatty acids and other acids or alkaline products which are often recycled within metabolic pathways (Horak et al., 2019, Pinu et al., 2017, Villas-Bôas, 2007). These metabolites are equally important to the individual microbes, to the whole microbiome community and to the host (Krautkramer et al., 2021, Horak et al., 2019). Microbial metabolites complement the host`s endogenous metabolism, and growing evidence suggests that they have an important role in modulating host physiology. Furthermore, bacterial metabolites can induce epigenetic modifications, that can either activate or inhibit the immune reaction, interfere with the host`s neuroendocrine system, can impact the intestinal mucosal surface, and are even able to reach the central compartment after passing the blood-brain-barrier (Connell et al., 2022, Bowland and Weyrich, 2022, Raimondi et al., 2021, Narengaowa et al., 2021, Dong et al., 2022). In the context of ELA and the seeding and establishment of the ELM it would appear logical that this early interaction is not only shaping immune tolerance, but also the microbial metabolome (Brandtzaeg, 2013).

Therefore, interest on the microbiome and its metabolites is now expanding to consider it as an environmental mediator and moderator of ELA associated diseases (Cox et al., 2022, Dube et al., 2009, Eriksson et al., 2014, Spitzer et al., 2013, Tomasdottir et al., 2015, Gern et al., 2009, Herzog and Schmahl, 2018, Mansuri et al., 2020, Wampach et al., 2018, Shao et al., 2019, Sarkar et al., 2021, Moore and Townsend, 2019, Elwenspoek et al., 2020, Elwenspoek et al., 2017c).

The concept of microbiota – immune – brain axis incorporates a multisystem host-microbe interaction, heavily affected by ELA, that includes epigenetic modifications, immune programming, metabolic programming and immunomodulatory activity of the microbiome (Holuka et al., 2020, Merz and Turner, 2021, Hajishengallis and Chavakis, 2021, Wei et al., 2021, Foster et al., 2021). Firstly, microbial metabolites provide a reservoir of substrates necessary for epigenetic modifications (Li et al., 2022). Secondly, epigenetic modifications are essential for immune programming at early life and for eliciting an immune response later on (Divangahi et al., 2021, Dutta et al., 2021, Ellmeier and Seiser, 2019). Thirdly, co-existence of host-microbe in the presence of highly specialised defence system against pathogens requires elegant organisation of host to differentiate from pathogenic to non-pathogenic taxa and the microbiome to modulate an unwanted immune reaction (Gensollen et al., 2016, Zheng et al., 2020, Belkaid and Hand, 2014). In fact, research on germ-free (GF) animal models exploited physiological and morphological differences in the absence of the microbiome and in particular defects in the development of lymphoid tissues and impairment of haematopoiesis (Gensollen et al., 2016, Belkaid and Hand, 2014, Sarkar et al., 2021, Wolfe and Markey, 2022). Clinical studies also suggest microbiome to influence development of lymphoid tissues and hematopoiesis via direct signalling on hematopoietic stem cells while alterations on the immunophenotype are resulting following interaction with microbial metabolites (Wolfe and Markey, 2022, Markey et al., 2020, Michonneau et al., 2019, Staffas et al., 2018, Liu et al., 2015). Growing evidence on the early life microbe-immune interaction suggests that commensal taxa travel to the thymus and are inducing expansion of T cells specific to the host`s microbiome (Zegarra-Ruiz et al., 2021).

In this study, we performed metabolic reconstructions on the OM relative abundance data of the EpiPath cohort which we have been previously reported to unveil the impact of ELA on the oral microbial metabolome (Charalambous et al., 2021). A subsequent

integration of the oral metabolome data with immunophenotype and lifestyle data aims to uncover mechanistic associations between oral microbial metabolites and the immune system.

4.2. Materials and Methods

Participants

In this study oral metabolome data were generated using our previously reported EpiPath cohort of 115 adults aged 20 to 25 years (Charalambous et al., 2021, Elwenspoek et al., 2017a, Elwenspoek et al., 2020, Elwenspoek et al., 2017c, Hengesch et al., 2018, Elwenspoek et al., 2017b). 73 control participants were brought up by their biological parents and 42 participants were adopted into Luxembourg from institutions worldwide. Median age at adoption was 4.3 months (IQR 0-15 months) (Elwenspoek et al., 2017b).

Oral samples

Saliva and buccal samples were used to generate oral microbial abundances as described by E.G. Charalambous et al (Charalambous et al., 2021). Briefly, saliva and buccal samples were collected using polyester swabs (Salimetrics, Cambridgeshire, UK) (Elwenspoek et al., 2020, Hengesch et al., 2018) and Isoxelix Buccal Swabs (Isohelix, Kent, UK) respectively. After extraction of microbial DNA using the "QiaGen DNA from body fluids kit" (Qiagen, Venlo, Netherlands), quantification (Qubit 1.2, Invitrogen, Merelbeke, Belgium) and quality assessment (Nanodrop, Thermofisher, Merelbeke, Belgium) the V4 region of the 16S gene was amplified and sequenced on the Illumina MiSeq system with v2 sequencing chemistry and 500 bp paired-end reads, as well as 10% PHIX control according to the manufacturer's protocol (Charalambous et al., 2021).

Metabolic reconstructions

Microbial metabolic reconstructions were performed using the COnstraint-Based Reconstruction and Analysis Toolbox tool (COBRA), AGORA2 as a the source of genome-scale microbial metabolic reconstructions and mgPipe from the Microbiome Modeling Toolbox (Heinken et al., 2023, Heirendt et al., 2019). The 16S generated microbial abundances were mapped onto AGORA2 at the genus level to generate the panGenus models (Heinken et al., 2023). Then, personalised microbiome community models were generated

for each participant from the 16S microbial abundances and the panGenus models using the mgPipe tool (Heinken and Thiele, 2022). Subsequently, Average European Diet parameters were introduced into the community models, generating individualised predictions of the oral microbiomes metabolic capacity based on each known exchange reaction at the genus and community level. These in silico models are based on maximum secretion and uptake fluxes of each microbial metabolite. All models were calculated in MATLAB version 2021a (Mathworks, Inc) with an IBM cplex 129 (IBM).

Bioinformatic and Statistical analyses

Further integration of metabolome and microbiome data into the immunophenotype and metadata as well as statistical analysis and visualisations performed with R (version 4.1.1 R Core Team, 2021) on R Studio Server (version 1.4.1717; R Core Team, 2021) (R Core Team, 2013, Allaire, 2012). R packages used overall are: dplyr, tidyverse, janitor, purrr, broom, rcompanion, ggplot2 and ggthemes (Wickham and Wickham, 2017, Wickham et al., 2019, Mangiafico and Mangiafico, 2017, Mailund, 2022, Arnold and Arnold, 2015, Wickham et al., 2016, Wickham et al., 2014, Robinson, 2014, Wickham, 2019). For data cleaning, we excluded metabolites present in less than 10% of the samples and metabolites with a standard deviation of 0 in order to exclude metabolites with no flux variation between study participants. Two types of statistical models were generated. Firstly, in order to detect group differences and also to identify covariates that drive the variance and are essential to correct for, we performed Wilcoxon Rank Sum Test, Fisher's exact test for $n>50$ or Welch T-test for $n<50$ as described below. Secondly we focused on regression series in order to assess the relationship between the independent variables or alternatively our metabolite flux as predictor of interest and other dependent covariates from our metadata. Here, we used generalized linear models on metabolic fluxes as response variable and the predictor of interest as immune cell counts or age of adoption. Data were log transformed when necessary. Covariate correction was applied to reduce the variance while increasing the statistical power for the detection of associations with the predictor of interests. For multiple testing correction, all p values were adjusted using the Benjamini-Hochberg (BH) Procedure (Benjamini, 2010).

4.3. Results

Personalised metabolic profiles for each EpiPath participant were generated following metabolic reconstructions based on microbial abundance profiles previously generated independently for both salivary and buccal communities (Charalambous et al., 2021). These microbial profiles contained 371 and 288 genuses respectively, representing a total of 24 phyla. All models were successfully processed using AGORA 2 panGenus models (a total of 424) from Cobra Toolbox with Matlab. (100 panGenus models for salivary community and 88 for buccal community). Overall coverage reached 77% for buccal samples and 70% for saliva samples.

4.3.1 Metabolic personalised models – overview

The simulated personalised metabolic profiles of each community for each participant generated complex profiles. These consisted of a total of more than 800 metabolites and more than 7800 reactions for both salivary and buccal communities. Overall, buccal models showed that adoptees had increased presence of reactions, metabolites and microbes compared to non-adoptees (statistic test: all FDR corrected $p<0.05$). Saliva models, however showed a homogenous presence of reactions, metabolites and microbes (test; all FDR corrected p-values>0.05).

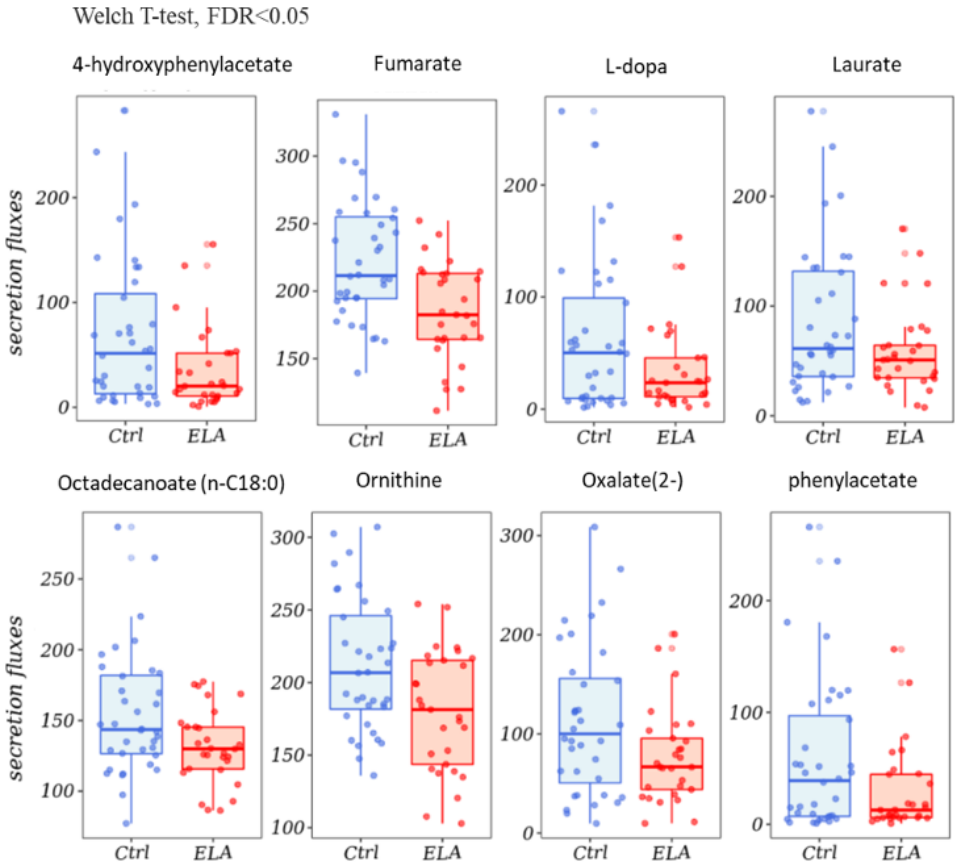


Figure 4.1. ELA changes the metabolome of the salivary microbiome.

4.3.2 ELA induces differences in the metabolic profiles of salivary and buccal bacterial communities.

4.3.2.1 ELA-induced differences

In initial pairwise comparisons we identified a total of 20 metabolites from both community-models that had a different secretion flux between adoptees and non-adoptees. Saliva models showed that 14 metabolites, from 10 different metabolic systems had a reduced secretion flux for the adoptees including: ethanol, fumarate, l-dopa, laurate, ornithine, oxalate(2-), phenylacetate, 4-hydroxyphenylacetate, 2-Hydroxyphenylacetate, 2,3-dihydroxicinnamic acid, 3-methyl-2-oxopentanoate, (3,4-dihydroxyphenyl)acetate, adenosine 3,5-bismonophosphate(4-), octadecanoate (n-C18:0) (Fig 1, Welch T-test BH<0.05). Similarly, buccal metabolic models revealed 6 metabolites from 6 different metabolic systems to have a difference in their secretion fluxes between our study groups,

including an increase in folate, glycine, citrate, lactose and spermidine and a reduction in methanol in the adoptees (Fig2 Fischer`s Exact, Mann-Whitney FDR<0.05).

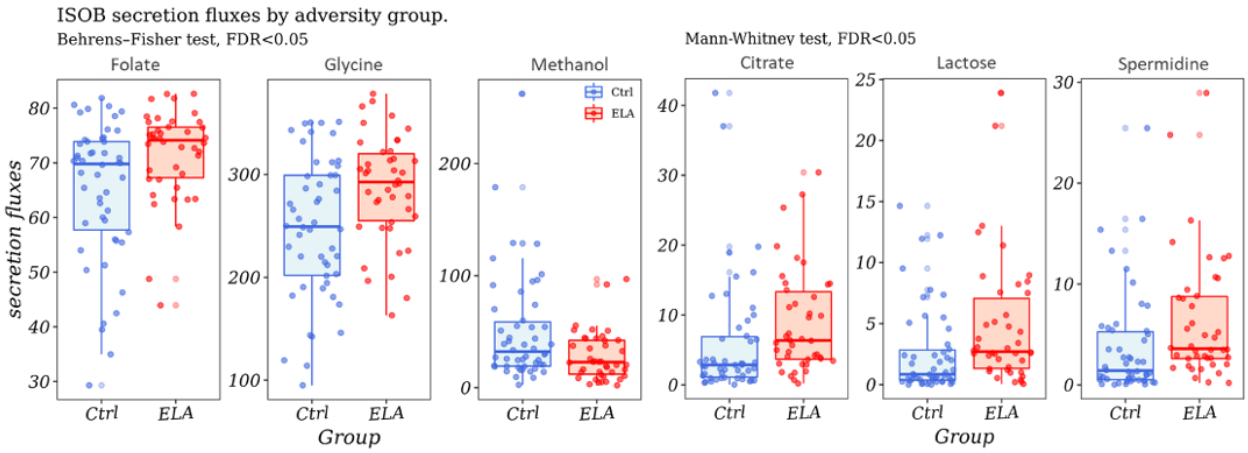


Figure 4.2. ELA changes the metabolome of the buccal microbiome.

4.3.2.2 The level of adversity contributes to metabolic changes

We used age of adoption as a proxy for the intensity of adversity experienced (Julian, 2013). Generalised linear models were used to assess whether the age of adoption, as a predictor variable, has an impact on the metabolic fluxes as a response variable. Three metabolites from the salivary models were positively associated with the age of adoption (D-Galactose, Lactose and N-acetyl-Glucosamine; Fig 4 A-C, GLM BH<0.05). Further sensitivity analyses were performed to rule out potential mediation by an additional factor and confirmed that the age of adoption is genuinely associated with the secretion flux of these metabolites. However, the physiological interpretion remains unclear.

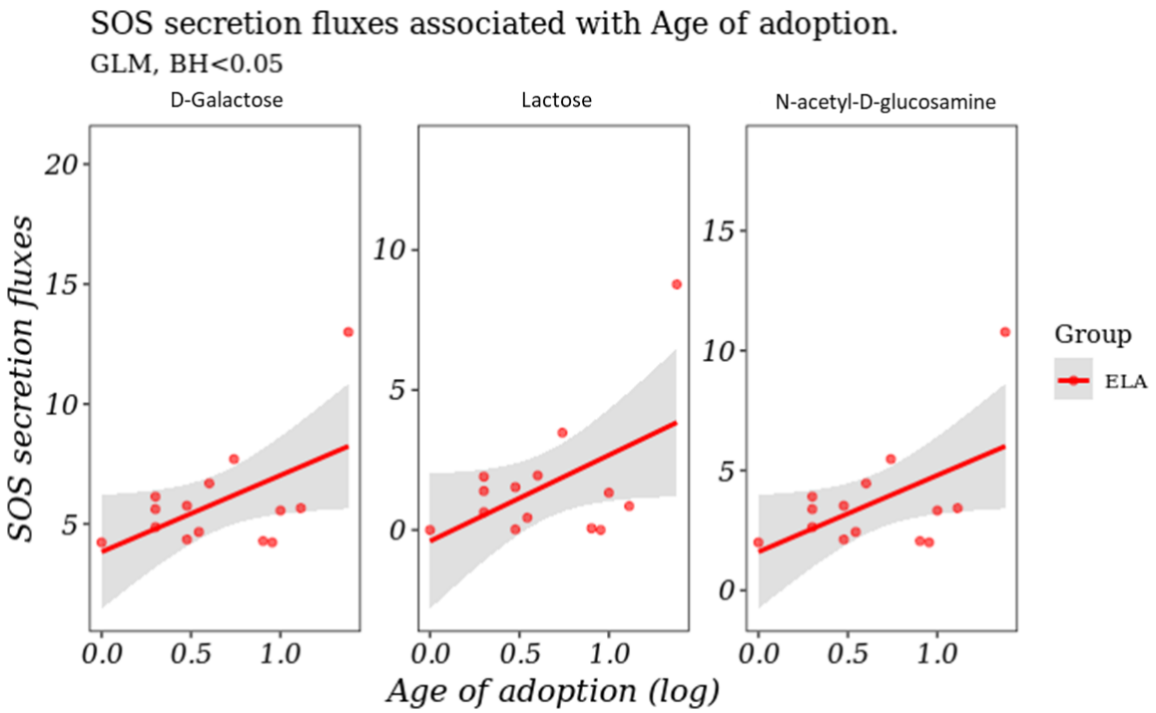


Figure 4.3. Age of adoption associates with the metabolic fluxes of 3 metabolites of the salivary microbiome metabolic models.

4.3.3 Identification of covariates that drive changes to the metabolic profiles

Readouts of the overall ELA phenotype from immune, microbial and hormonal systems have all been shown to depend partially on a series of covariates including sex, contraception, smoking and *herpesviridiae* exposure. Here we test the exact contribution of these covariates to differences in the microbial metabolic profiles.

4.3.3.1 Sex and oral contraceptive use

The buccal models revealed Ubiquione-8 to have an increased secretion and uptake flux in males compared to females (Fig S2, Wilcoxon BH<0.05). In addition, females taking oral contraceptives showed an increased secretion flux of octadecanoate (n-C18:0) and a reduced secretion flux for p-Cresol as well as an increased uptake flux of hexadecanoate (n-C16:0) (Fig S2, Wilcoxon BH<0.05).

Similarly saliva models showed that secretion fluxes of D-glutamate and Succinylbenyoate were increased in males, while Butyrate was increased in females (Fig S2, welch t-test BH<0.05). Furthermore, L-cysteinyglycine showed a reduced uptake flux for females taking oral contraceptives while nicotinamide mononucleotide (NMN) showed an increased uptake flux (Fig S2, welch t-test BH<0.05). For both buccal and salivary models these interactions did not depend upon exposure to adversity.

4.3.3.2 **Smoking**

As lifestyle has a pivotal role on the development of the microbiome as well as its functional capacity, we assessed the impact of smoking on the oral microbiome metabolic profiles (binary: smokers vs. non-smokers). A total of 21 metabolites appeared to have a difference on their secretion flux between smokers and non-smokers in both buccal and saliva models. Buccal models revealed 18 metabolites with reduced secretion flux in smokers: 2,3-dihydroxicinnamic acid, 2-hydroxyphenylacetate, 3alpha,12alpha-dihydroxy-7-oxo-5beta-cholanate, 4-hydroxyphenylacetate, 5-Methylthioadenosine, 7-dehydrochenodeoxycholate, allantoin, butyrate, D-arabinose, D-gluconate, dopamine, hydrogen peroxide, laurate, methanethiol, O2, oxalate(2-) and thiamin (Fig S2, wilcoxon BH<0.05). Furthermore, L-proline was found to be increased in the buccal models (Fig S2, wilcoxon BH<0.05). Saliva models revealed 2 metabolites that showed a decreased secretion flux in smokers: Cob(I)alamin and tyramine; and 1 metabolite: pyridoxal 5-phosphate had an increased secretion flux in smokers (Fig S3, welch t-test BH<0.05). As for sex and contraception, these did not depend upon exposure to adversity.

4.3.3.3 ***Herpesviridiae*** **infections**

Viral infections are known to have a strong association with adversity (Elwenspoek et al., 2017c) as well as the oral microbiome (Charalambous et al., 2021). Consequently, we tested the association between metabolic profiles of the oral microbiome and anti-EBV, HSV and CMV antibody titres. In our buccal models we identified 26 metabolites whose secretion or uptake flux was dependent upon anti-EBV antibody titres, confirming the importance of prior viral exposure. EBV positive individuals showed an increase secretion flux of; 2-Demethylmenaquinone 8, L-dopa, 3-methyl-2-oxopentanoate, 4-hydroxyphenylacetate, S-Adenosyl-L-homocysteine, Allantoin, D-Arabinose, Biotin, L-cysteine, laurate, D-Glutamate, Hydrogen sulphide, Nitrogen, Nitrous oxide, Nitric oxide, Nitrite, oxalate(2-), Siroheme and uracil (fig3, wilcoxon BH<0.05). An increase in the uptake flux of metabolites; 1,2-Diacyl-sn-glycerol (dioctadecanoyl, n-C18:0), 2-Demethylmenaquinone 8, linoleate, L-phenylalanine, Siroheme, was also observed in seropositive individuals (fig4, wilcoxon BH<0.05). In addition, reduced glutathione showed a reduction on it uptake flux in seropositive individuals (fig4, wilcoxon BH<0.05). Prior HSV exposure affected both buccal and salivary microbial metabolomes. In buccal metabolic models, 2 metabolites: L-arabinitol and Pyridoxamine showed an increased secretion flux in seropositive individuals; while Menaquinone 7 and 4-Aminobenzoate showed an increased uptake flux (fig S4, Wilcoxon BH<0.05). Saliva metabolic models revealed 5 metabolites: D-ribose, L-isoleucine, L-leucine, L-proline and Urea that have a reduced secretion flux in seropositive individual (fig S4, welch T-test BH<0.05). Prior exposure to CMV induced differential secretion flux between seropositive and seronegative individuals from buccal community models for 14 metabolites. These metabolites are 2-phenylethanamine, Citrate, D-Galactose, D-Glucose 6-phosphate, Isobutyrate, Isocaproate, Isovalerate, Lactose, L-Norvaline, maltotriose, N-acetyl-D-glucosamine, N-acetyl-D-mannosamine, n-butylamine and Pyridoxal 5-phosphate (fig S4 Wilcoxon BH<0.05).

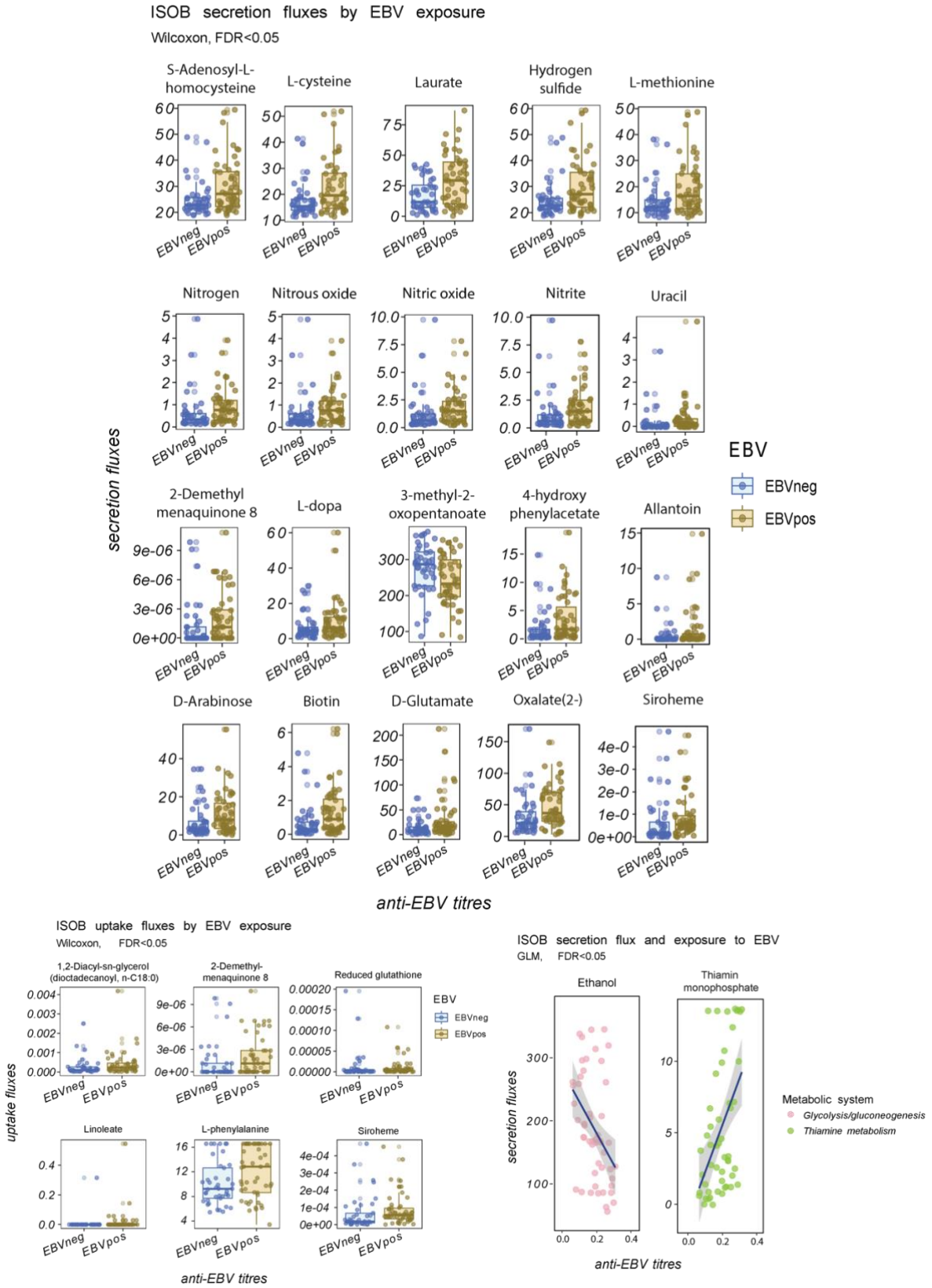


Figure 4.4. Prior exposure to EBV infection changes the metabolic profile of the buccal microbiome metabolic models.

As our suspected covariates had a significant effect on metabolite fluxes in our binary analyses above, we used age, sex, BMI, smoking, corrected generalised linear models using viral antibody titres as the dependent variable. A total of 4 metabolites; ethanol and thiamine monophosphate from buccal metabolic models demonstrated a negative and a positive association respectively whereas, L-glutamine and Urea from salivary metabolic models, revealed a negative association between the metabolite secretion flux and the anti-EBV antibody levels (fig4 A-D, GLM BH<0.05). In contrast to anti-EBV titres, Anti-HSV and anti-CMV antibody levels showed no association with the metabolic fluxes.

4.3.4 Regression and mediation models of the immune-metabolome interactions

Having identified the metabolic changes induced by early life adversity, we made a series of generalised linear models integrating the metabolic fluxes from the salivary and buccal metabolic models with our previously published immune system profiling (Elwenspoek et al., 2017c, Elwenspoek et al., 2017a). Based on our identification of the key metabolic covariates, and in order to reduced residual variance, all models were corrected for age, sex, BMI, smoking, and exposure to EBV, HSV and CMV. From the full dataset of 48 immune populations we identified 5 significant associations, most importantly for T cells and NK cells.

4.3.4.1 Association with CD4-CD8 T cell Immunosenescence

T-lymphocyte immunosenescence is a consequence of early-life adversity that we have shown to be associated with specific taxa of the oral microbial communities (Charalambous et al., 2021). Consequently, we looked for potential associations between microbial metabolites and senescence of the principal T-lymphocyte subsets. Circulating senescent CD4 cells associated with the secretion flux of 4 metabolites originating from 4 metabolic systems in both community models. Hydrogen secretion flux showed a positive association and ethanol had a negative association with CD4CD57 cell numbers in the salivary metabolic models (Fig 5, GLM BH<0.05). Biotin and L-glutamine showed a positive and a negative association with CD4CD57 respectively in buccal metabolic models (Fig 5, GLM BH<0.05).

CD8 T-cells were previously reported to be significantly associated with CMV (Elwenspoek et al., 2017a) but we found no associated taxonomic markers from the oral microbiome (Charalambous et al., 2021). Nevertheless, circulating senescent CD8 cell numbers were associated with the secretion flux of 9 metabolites originating from 9 metabolic systems in both community models, buccal and salivary. From salivary metabolic models, Acetoacetate, D-alanine, ethanol and ornithine showed a negative association while D-Galactose showed a positive association with CD8D57 cell counts (Fig 5, GLM BH<0.05). From buccal metabolic models, Biotin, D-Glucosamine, S-Adenosyl-homocysteine and Ubiquinone-8 demonstrated a positive association with CD8CD57 cell numbers (Fig 5, GLM BH<0.05).

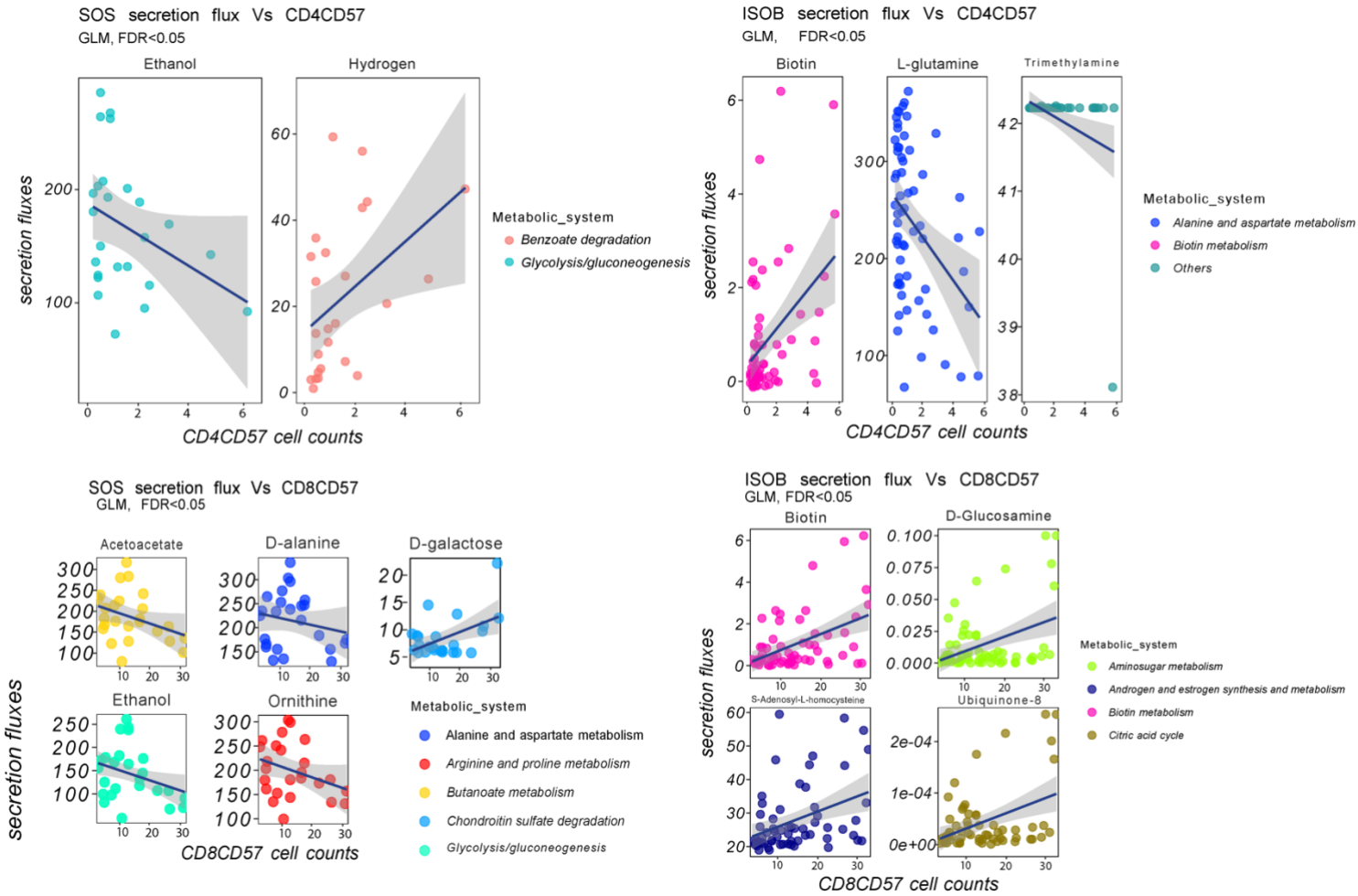


Figure 4.5. Expression of CD57 senescence marker on CD4 and CD8 Tcells associates with metabolites from both salivary (SOS) and buccal (ISOB) microbiome metabolic models.

Interestingly, uptake fluxes of 2 metabolites from the salivary metabolic models showed an association with the CD57 expressing CD8 cell numbers: ethanol was negatively associated and octadecanoate (n-C18:0) was positively associated. Buccal metabolic models also had 4 metabolites whose uptake fluxes were found to be associated with CD57 expressing CD8 cell counts. These were ethanol that was negatively associated and the positively associated D-Glucosamine, L-cysteinyglycine and ubiquione-8 (GLM BH<0.05).

4.3.4.2 Association with Th17 cell numbers and activation status

Th17 cells, pro-inflammatory T helper cells characterised by their unique capacity in producing IL-17, are specialised cells involved in immune protection against various microbes, particularly in the oral cavity. As such, we searched for possible associations between microbial metabolites and the total number and activated Th17 cells. Using covariate controlled GLMs we identified 6 metabolites with reduced secretion fluxes in the buccal metabolic models that negatively associated with CD69 expressing Th17 cells from 4 metabolic systems; D-alanine, D-lactate, fumarate, glycine, propionate and thymidine (Fig 6, GLM BH<0.05).

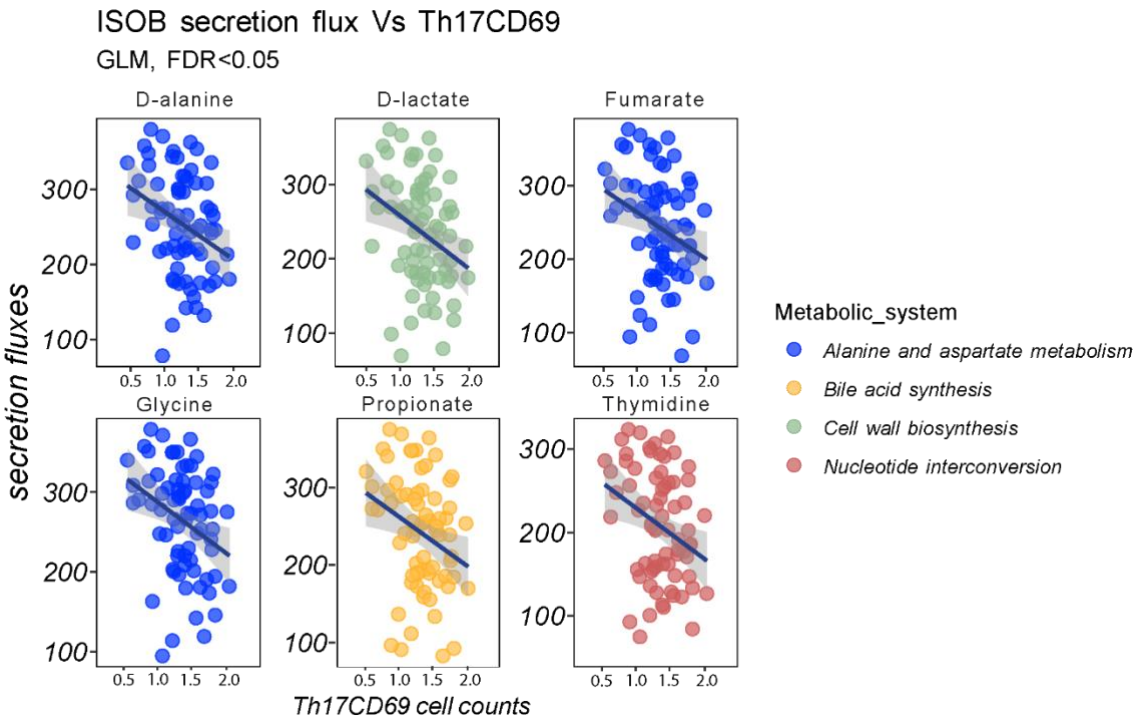


Figure 4.6. CD69 activation marker on Th17 cells associates with metabolites from the buccal microbiome metabolic models.

4.3.4.3 Association with Natural Killer cell numbers and activity

Innate cells such as natural killer cells (NKs) are the first line of defence and often interact with commensal bacteria. Adoptees in this cohort showed decreased cytotoxicity on their NK cells (Fernandes et al., 2021) while associations between bacterial taxa of the oral microbiome of the cohort and total numbers of NK cells and activated NK cells have been previously reported by Charalambous et al. When we investigated the relationship between microbial metabolic fluxes and activated NK populations with both buccal and salivary metabolic models we identified a series of associations (fig 7, GLM BH<0.05).

From the salivary metabolic models, 2 metabolites, myo-inositol and thiamine monophosphate showed a negative association between their secretion flux and circulating numbers of activated CD69 expressing NK cells while uptake flux of octadecenoate (n-C18.1) was negatively associated with activated CD69 expressing NK cells (fig 7). From the buccal metabolic models, secretion fluxes of 7 metabolites from 5 metabolic systems were identified that showed a positive association with activated CD69 expressing NK cells; (3,4-dihydroxyphenyl)acetate, 2,3-dihydroxicinnamic acid, 4-hydroxyphenylacetate, D-gluconate, keto-phenylpyruvate, phenylacetate, tetradecanoate (n-C14:0) (fig 7, GLM BH<0.05). Uptake flux of an additional metabolite, 4-aminobenzoate, also showed a positive association with the activated CD69 expressing NK cell counts (fig 7, GLM BH<0.05). Further mediation analysis showed that male biological sex is the mediator behind the association of (3,4-dihydroxyphenyl)acetate, 2,3-dihydroxicinnamic acid, 4-hydroxyphenylacetate, D-gluconate and 4-aminobenzoate (fig 7, GLM BH<0.05).

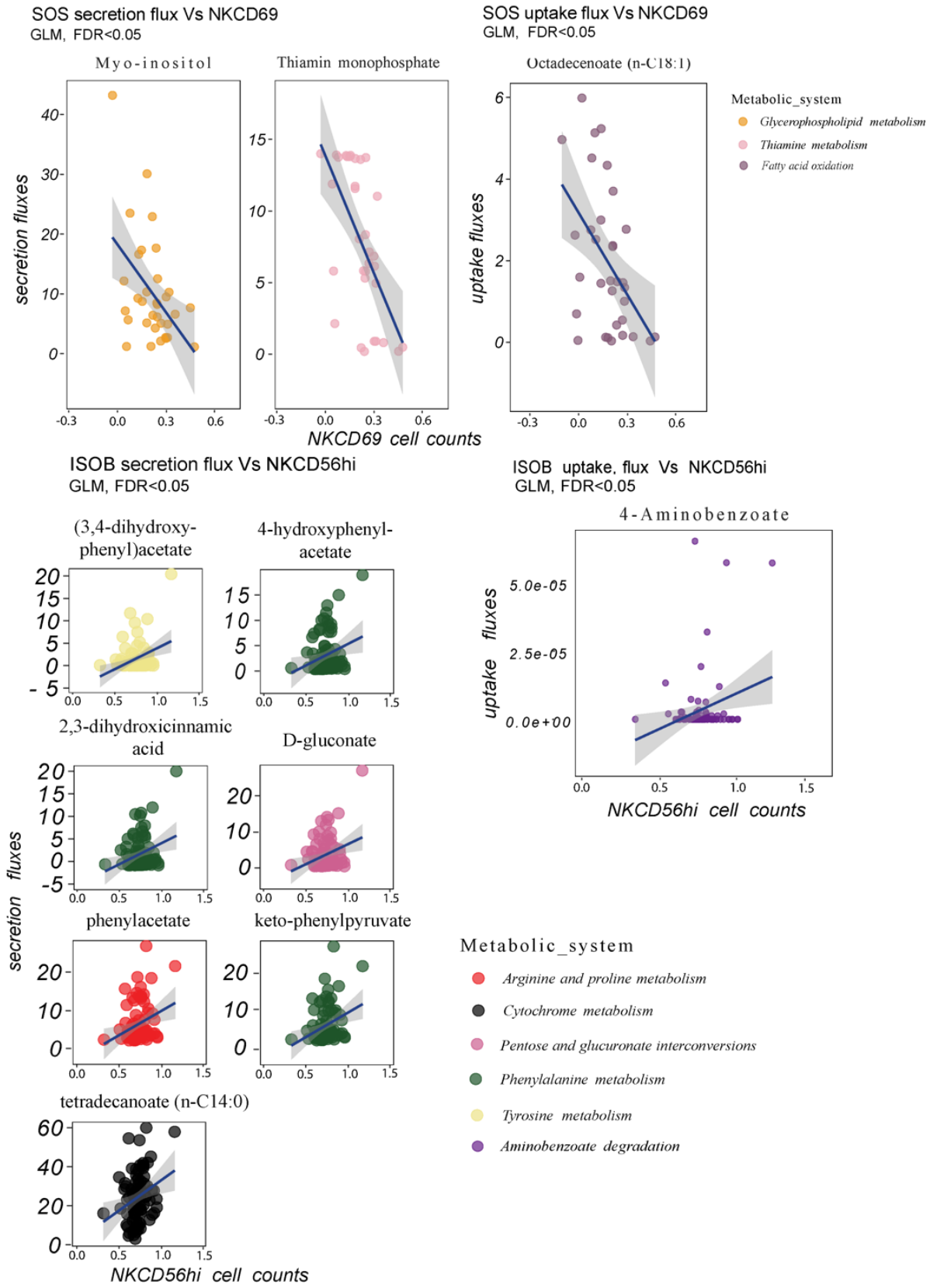


Figure 4.7. Activation markers CD69 and CD56hi on NK cells associate with metabolites from the salivary (SOS) and buccal (ISOB) microbiome metabolic models respectively.

4.3.5 Metabolic ratios are affected by adversity and associate and correlate with immune cell populations

Complementary to metabolic associations, the ratio between two single metabolites can also point towards a particular phenotype or may also associate to various biological markers such as activated immune cells and their functional status (Dutta et al., 2021, O'Neill et al., 2016, Kok et al., 2018, Molzer et al., 2021, Carriche et al., 2021, Guan et al., 2021, Schulthess et al., 2019, Woo and Alenghat, 2022, Gerhauser, 2018, Amatullah and Jeffrey, 2020, Wu et al., 2021).

From the buccal and salivary metabolic profiles of EpiPath, we calculated the ratio between a series of key metabolites that were subsequently used as a response variable in our statistical models. Metabolite ratios were calculated for: butyrate/acetate, acetate/pyruvate, Fe^{2+}/Fe^{3+}, folate/S-adenosyl-L-methionine, S-adenosyl-L-methionine/S-adenosyl-L-homocysteine (Amet/Ahcys or SAM/SAH), succinate/fumarate, trimethylamine/trimethylamine N-oxide (Tma/Tmao), linoleate/alpha-linolenate, $Copper^{2+}/Calcium^{2+}$.

From all the calculated ratios, salivary metabolic profiles revealed that Succinate/Fumarate ratio was increased in the adoptees (fig 8 A, Welch T-test BH<0.05) and in the buccal metabolic profiles Tma/Tmao ratio was also increased in the adoptees (fig 8 B, Wilcoxon Rank Sum Test BH<0.05). Additional association analyses using our generalised linear revealed one association from the buccal metabolic profiles. Here, the Tma/Tmao ratio showed a negative association with CD69 expressing granulocytes (fig 8 C, GLM BH<0.05). Salivary metabolic models showed no associations.

A further series of correlation analyses demonstrated that both buccal and salivary metabolic models had important correlations between metabolic ratios and immune cells activation status and functionality. Butyrate/Acetate ratio from salivary metabolic models showed a strong negative correlation with naïve CD4CD57 cells as well as activated CD69 expressing NK cells (fig 8 D, Spearman`s correlation BH<0.05). Succinate/Fumarate was negatively correlated with activated CD11b expressing monocytes (fig 8 D, Spearman`s correlation BH<0.05). Acetate/Pyruvate ratio from buccal metabolic models had a negative correlation with CD56hi NK cells as well as senescent (CD57 positive) CD4 and CD8 cells (fig 8 D, Spearman`s correlation BH<0.05). The buccal S-Adenosyl-L-methionine/S-

Adenosyl-L-homocysteine (Amet/Ahcys or SAM/SAH) ratio had a strong negative correlation with CD69 expressing Th2 cells, a strong positive correlation with CD4 CD57

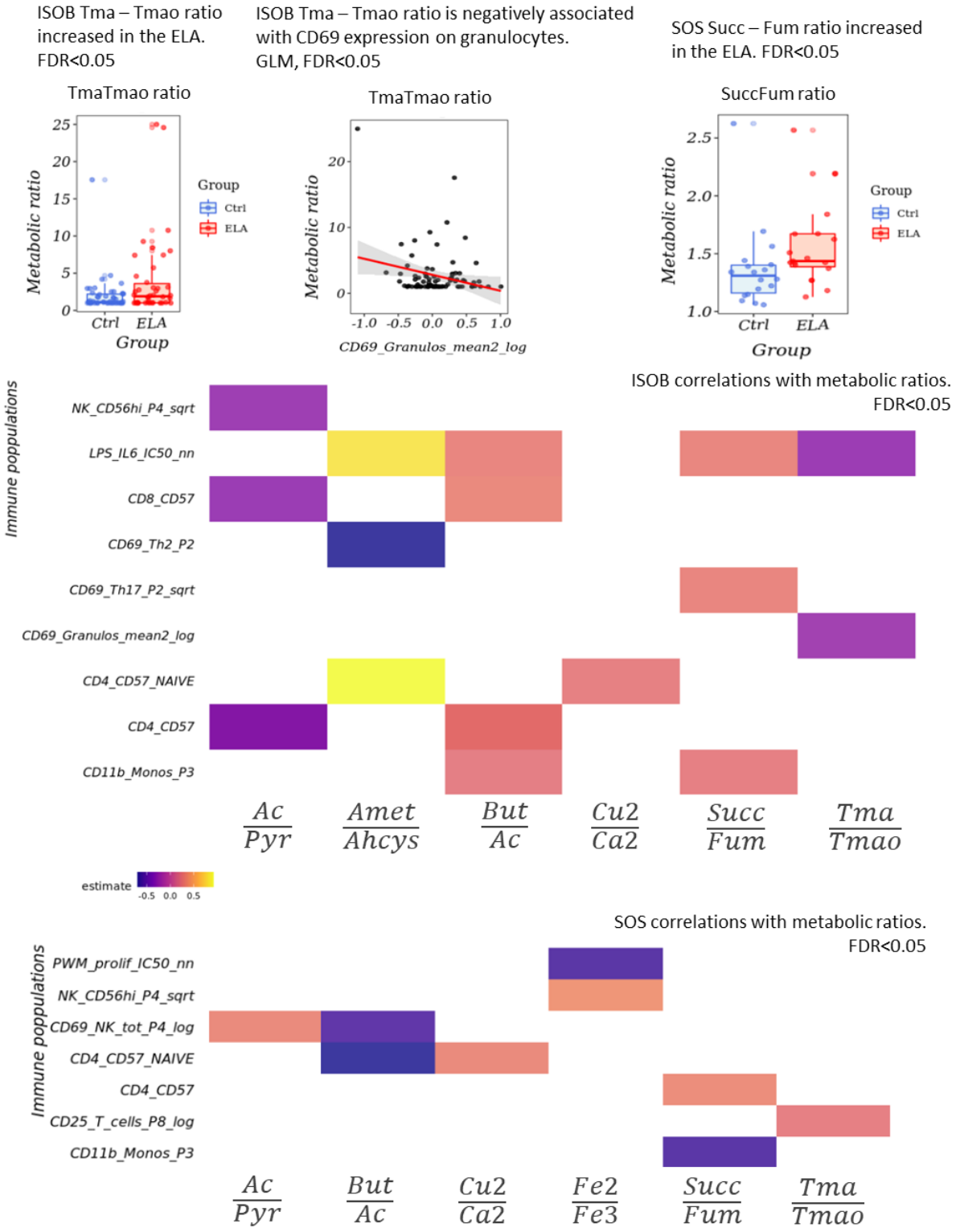


Figure 4.8. Metabolic ratios of oral microbiome are affected by adversity and associate and correlate with immune cell populations.

cells and IL6 production following LPS stimulation in whole blood (fig 8 D, Spearman`s correlation BH<0.05). In addition, functional test for glucocorticoid receptors in B-cells and monocytes also correlated with metabolite ratios in both community metabolic models. In salivary models, the Fe2/Fe3 ratio had a strong negative correlation with pokeweed mitogen (PWM) induced proliferation of B-cells (fig 8 D, Spearman`s correlation BH<0.05). Similarly, in buccal models, the Trimethylamine/Trimethylamine N-oxide (Tma/Tmao) ratio also correlated negatively with LPS induced IL6 production from B cells and monocytes as well as numbers of activated CD69 granulocytes (fig 8 D, Spearman`s correlation BH<0.05). Of all the metabolite ratios, Tma/Tmao in buccal models was particularly interesting, as it was the most connected to the immune system. In particular, in our data following our diverse statistical model, Tma/Tmao ratio associated and correlated with activated CD69 granulocytes (fig 8 D, Spearman`s correlation BH<0.05).

4.4. Discussion

In this study we have identified differences on the microbial metabolites of the oral microbiome up to 20 years following ELA. Our results show differences in metabolic fluxes between adoptees and non-adoptees, differences between smokers and non-smokers, differences based on sex, contraception as well as exposure to early life infections with herpesviridiae. In addition, we detected associations between the OM microbial metabolites and activation status of immune cell populations as mechanistic links to the previously identified observations with the taxonomic composition of the OM.

Our findings release the first evidence of the functional impact the ELA has on the ELM, detectable on the adult OM in particular in humans. A similar concept study with a different ELA model by Coley et al., 2021, also reported ELA affecting gut microbiome metabolites (Coley et al., 2021). Moreover buccal microbial metabolites that were increased in the adoptees such as folate and spermidine are very important for epigenetic modifications, regulation of T cell differentiation and cell viability (Kim et al., 2021, Pietrocola et al., 2015, Seiler and Raul, 2005). This raises the question: is the microbiome programmed to modulate the host in order to reach homeostasis? In contrast, salivary L-dopa was found decreased in the adoptees and potentially explains the ELA psychological phenotype, as L-dopa is precursor for Dopamine (Wu et al., 2021). However a decrease

secretion flux of Dopamine`s precursor it is not sufficient to draw conclusions. Taken together, results from these studies expand the need of precise design and longer term investigations on ELA-microbiome-metabolome including different ELA models and metabolome of oral and gut microbiomes.

Our results also demonstrate how the microbial metabolism is changing by host`s sex and usage of contraceptives, in contrast to our previous findings where on the EpiPath we did not see sex-dependent taxonomic differences. This highlights that functional impact is not always dependent on the taxonomic profile of the microbiome. In addition, our results also represent the impact of lifestyle factors such as smoking habits and prior exposure to *herpesviridiae* infections. The initial colonisation and organisation of the microbiome is dependent on the host`s genetics whilst its further compositional development and metabolic programming depends on the early life environment and external stressors (Brandtzaeg, 2013, Shaw et al., 2017, Premaraj et al., 2020a, Stahringer et al., 2012). This, lead to the hypothesis that the early life environment shapes the taxonomic composition by driving the metabolic programming of individual community members.

Following our previously published observational associations of the taxonomic composition with the EpiPath covariates, our metabolomic results continue to mirror the EpiPath phenotype and covariate structure. The numerous associations and correlations that we report give the first mechanistic evidence of the microbiome-immune crosstalk observed in this cohort. For instance, ethanol has been reported to dysregulate differentiation of CD4 T cells and in our data ethanol negatively correlates with senescent CD4 T cells and CD8 T cells (McTernan et al., 2022). Similarly, glutamine is broadly popular as a metabolic fuel and also essential for protein synthesis, various signalling pathways and gene expression (Paixao et al., 2021, Cruzat et al., 2018). Moreover, in EpiPath glutamine showed a negative association with the senescent CD4 T cell supporting the results of Paixao et al., 2021, where supplementation of glutamine in an elderly, immunosenescence characterised population, resulted to an increase in the salivary inflammatory cytokines (Paixao et al., 2021). Furthermore, we reported ornithine to negatively associate with senescent CD8 T cells and in vitro studies suggest that this metabolite selectively suppresses the development of cytotoxic effector T cells (Droge et al., 1985, Janicke and Droge, 1985, Susskind and Chandrasekaran, 1987). An immunotherapeutic similar to fumarate was reported by Wu et

al., 2017, to inhibit the activation of Th17 cells (Wu et al., 2017b). In our results, higher secretion of fumarate associated with lower cell counts of CD69 expressing Th17 cells.

Our results on the microbial metabolites modulating activation status of immune cells and the impact of ELA on the metabolic capacity of the oral microbiome provide the first mechanistic link on how the ELA-OM interaction result to phenotypic changes. Pre-existing evidence suggest that the microbial metabolites can interact with immune cell progenitors directly from the bone marrow (Woo and Alenghat, 2022). Particularly, animal model data demonstrated segmented filamentous bacteria (SFB) microbial metabolites to promote methylation-demethylation on bone marrow derived immune progenitors shaping the host`s immune profile (Woo and Alenghat, 2022, Burgess et al., 2016, Burgess et al., 2020). The remaining question for our ELA model concerns the temporality of this interaction. Does this only happen during the early life development of the immune system or is this an ongoing life-long interaction between the ELA phenotype, the bone marrow stem cells, and the OM?

Despite the mechanistic approach used in our study, our in silico design unfortunately has limitations. The metabolic reconstructions are based on 16S data. This restricted our metabolic community models to the genus level. Using the panGenus models is a revolutionary approach, not used before in this type of investigation and can be very accurate for certain genera, reactions and metabolic systems whilst broad for some others. Furthermore, our cohort includes “only” 115 participants of which microbiome data of the 98 were possible to successfully model. Even though this is a substantial sample size for an ELA study it is considered small for a microbiome and metabolomics study. In addition, due to the nature of the cohort, there is lack of metadata important for microbial metabolite research including diet habits and oral health status. Knowledge of particular diet habits would have reinforce our simulations and information on the oral health of the participants could guide us to a more targeted approach (Dimitrov and Hoeng, 2013). Nevertheless, we have previously demonstrated that despite the limitations of 16S sequencing, we were able to see the ELA impact on the OM composition and metabolic capacity that proves ELA having a long-term effect on the microbiome. Subsequently, we prove how the microbial metabolites are see-sawed as an action-reaction consequence of the ELA-OM interaction.

4.5 Conclusion

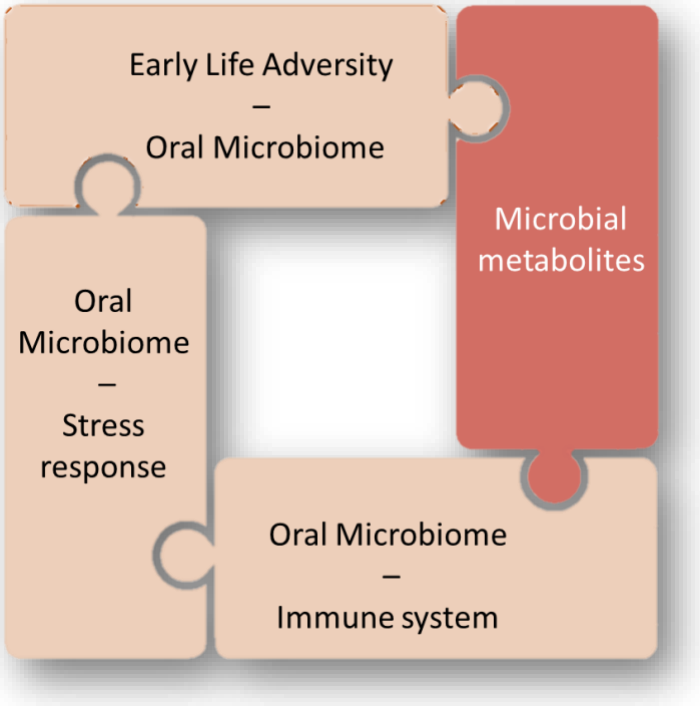
Our data clearly demonstrate a link between ELA and the oral microbial metabolome. Despite ELA occurring at a very early age, we detected interaction with the host 20 years later. The two oral communities investigated demonstrated associations with microbial metabolites from both communities to the presence of immunosenescent CD4 and CD8 T-cells, with the activation status of Th17 cells and natural killer cells. Associations were also identified with prior exposure to a *herpesviridiae* infection. Our results are the first evidence of a mechanistic explication of previously reported microbiome – immune system interactions. Our data suggest the involvement of different metabolic reactions and pathways which ELA results to long term taxonomic and functional changes in the oral microbiome with the involvement of the immune system.

Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Comité National d'Ethique de Recherche (CNER, reference 202004/01) and the Ministry of Health (Luxembourg, reference 831x6ce0d), and is registered on ClinicalTrials.gov (NCT 04379297).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study

Chapter 5: General Discussion – Assembling the jigsaw

This section condenses key findings from previous Chapters 2-4, where by constructs the jigsaw with the ELA-OM, OM-stress response, OM-immune system bidirectional interactions and the microbial metabolites being the key players.



What drives the interactions between ELA, OM, immune system and stress?

The first ever microbiome paper was published in 2002 and interest on microbiome research has only emerged over the last 2 decades with the larger proportion concentrated on the gut microbiome (Shanahan, 2002). Interestingly, the oral microbiome has been in the centre of interest of oral health experts since 1971 (Socransky and Manganiello, 1971) and it is only within the last decade we are seeing its importance in the context of systemic health. Here, I attempted to explore host-microbe interactions in the same ELA-institutionalisation model throughout the thesis. The results of this investigation have been described in Chapters 2, 3 and 4.

According to Chapter 2, the first aim of this Thesis was to detect if ELA leaves an impact on the OM. Indeed, my data demonstrate the presence of a clear link between ELA and the OM detectable in later adulthood. Given the opportunity to compare two oral communities, I showed that their compositions associate to each other and yet remain distinct from each other. I also revealed that the OM is not only linked to ELA, it also interacts with the immune system. I showed that from a previous report where ELA induced higher activation and senescence of the immune system, the oral taxa of the same individuals associated with this immunosenescence observed on CD4 T cells. In addition, I also extended the evidence of this interaction with the reporting of oral taxa associating with the circulating numbers and activation status of NK cells. Furthermore, I also showed that this interaction of the OM with the immune system is complicated by showing how the oral composition is depended to prior exposure of a *herpesviridae* infection.

Nevertheless, these remain observations of the multivariate relationship and diverse interactions the OM has with ELA and immune system functional status persisting long-term taxonomic changes. A summary of the key findings of Chapter 2 are listed below:

5.1 Chapter 2 key findings: OM – ELA – immune system

- Salivary and buccal microbial communities are distinct niches both consisting the OM
- Taxonomic differences in the OM depended on institutionalisation, detectable 24 years after
- Taxonomic differences in the OM related to smoking habits
- Taxonomic differences in the OM related to senescent CD4 T cells

- Taxonomic differences in the OM related to circulating numbers and activated status of NK cells
- Taxonomic differences in the OM related with prior exposure of *herpesviridiae* viruses

The intense psychophysiological stress of ELA also affects the HPA axis activation and downstream stress response commonly known as "fight or flight" response upon exposure to a stressor. In parallel, it has been proven that stress can drive dysbiosis in the microbiome. According to Chapter 3, the aim of this 2nd study was to explore how the microbiome reacts and interacts with the stress response of their host. With the inclusion of both stress-induced cortisol measurements and stress-induced glucose measurements together with the cohort`s oral microbial composition I was able to tackle my aim. Three OM members from the buccal community together with 2 members from the salivary community showed to alter the stress-induced cortisol response. Concurrently, 8 OM taxa from buccal community interacted with the stress induced glucose response. In particular, their presence, absence and even presence in increased abundance determined the kinetics of glucose following seCPT exposure. Overall I demonstrated that the oral interferes with the activation of HPA axis following exposure to an external stressor. As described in Chapter 3, key findings are listed below:

5.2 Chapter 3 key findings: OM – stress

- OM interacts with stress response following HPA axis activation
- Presence of 2 salivary taxa of the OM alter the kinetics of stress-induced cortisol
- Presence of 3 buccal taxa of the OM define the kinetics of stress-induced cortisol
- Presence of 3 buccal taxa of the OM define the kinetics of stress-induced glucose
- Difference in the relative abundance of 6 buccal taxa of the OM define the kinetics of stress-induced glucose
- High relative abundance of 6 buccal taxa of the OM delay the clearance of stress-induced glucose

Following these observational results, I wanted to find the mechanisms behind these interactions. Pre-existing literature highlights that the microbial metabolites supplement the host`s metabolism and can modulate the physiology of their host by inducing epigenetic modifications (Connell et al., 2022, Bowland and Weyrich, 2022, Raimondi et al., 2021,

Narengaowa et al., 2021, Dong et al., 2022). Hence, taking the concept of microbiota – immune – brain axis combining a multisystem host-microbe interaction, I hypothesized that the missing piece of the puzzle is metabolism and in particular the microbial metabolites. To challenge this hypothesis I performed metabolic reconstructions on the OM composition for each salivary and buccal communities.

According to Chapter 4, the first aim of this 3rd study was to detect if ELA shapes the metabolome of the OM. Truly, my data from the *in silico* metabolic modelling showed that ELA contributed to metabolic differences detected on the metabolome of the OM. I initially identified differences on both salivary and buccal microbial metabolites of the oral microbiome, 20 years after exposure to ELA. My results revealed the adoptees and non-adoptees to have different metabolic profiles of their OM. Interestingly, the measure of severity of adversity, age of adoption, contributed in these metabolic changes. The 2nd aim of this 3rd study was to detect the impact of a set of lifestyle and environmental covariates on the metabolome of the OM. I showed that sex, contraception, smoking and prior exposure to *herpesviridiae* changes the metabolic profile of the OM. Subsequently the 3rd and last aim of this study was to assess the capacity of these microbial metabolites to interfere with immune system functioning. I unveiled various microbial metabolites from both salivary and buccal OM to associate with senescence of CD4 and CD8 T cells. In addition a number of buccal microbial metabolites negatively associate with activation status of Th17 cells whilst another set of microbial metabolites positively associated with the activation status of NK cells. Furthermore, a few salivary microbial metabolites negatively associated with the activation status of NK cells. Lastly, knowing these metabolites are often recycled in metabolic pathways and converted to other downstream metabolites, I considered the metabolite ratio of key metabolites. This approach reinforced the previous findings as I identified metabolite ratio from both OM communities, involved in immune activation pathways to differ between adoptees and non-adoptees. Additionally, I identified a buccal metabolite ratio to negatively associate with the activation status of granulocytes. Lastly, a mixture of 8, salivary and buccal metabolite ratios correlated with immune activation markers. Overall, Chapter 4 provides the first mechanistic evidence of a gut-microbe interaction in our ELA-OM study design via the OM microbial metabolites. These mechanisms focus on the regulation of immune cell activation by the OM. As described in Chapter 4, key findings are listed below:

5.3 Chapter 4 key findings: OM microbial metabolites – ELA – immune system

- OM salivary and buccal niches harbor different metabolomes
- ELA shapes the metabolic profile of the salivary microbiome
- Age of adoption contributes to the changes detected on the metabolome of the salivary microbiome.
- Sex alters the metabolic profiles of the OM salivary and buccal communities
- Contraception alters the predicted metabolic profiles of the OM salivary and buccal communities
- Smoking alters the predicted metabolic profiles of the OM salivary and buccal communities
- Prior infection by *Herpesviridiae* alters the predicted metabolic profiles of the OM salivary and buccal communities
- Buccal and Salivary microbial metabolites interfere with senescence in CD4 and CD8 T cells
- Buccal microbial metabolites interfere with activation status of Th17 cells measured by CD69 immune activation marker
- Salivary microbial metabolites interfere with activation status of NK cells measured by CD69 immune activation marker
- Buccal microbial metabolites interfere with activation status of NK cells measured by CD56 immune activation marker
- Metabolic ratios of oral microbiome are affected by adversity and associate and correlate with immune cell populations

In summary, findings from Chapters 2 and 3 provide the observational evidence of an ELA-OM-immune system – stress interaction and findings from Chapter 4 disclose the first mechanistic evidence of the OM using microbial metabolites to interact with ELA and the host`s stress and immune responses.

5.4 Overall strengths and limitations

The findings described above from Chapters 2, 3 and 4 are evidence generated using samples and data from the same cohort, EpiPath, that investigates the impact of ELA in an institutionalisation model. The biggest limitation of this project was the limited quantity of biobanked samples that only allowed me to perform 16S sequencing to ensure good-quality data. As a result, I have a limited taxonomic resolution in comparison to other metagenomics studies. Consequently, the metabolic reconstructions I performed are based on 16S data only allowing me to model the metabolism of the OM to the genus level. An approach that is considered revolutionary and was never used before in this type of investigation meaning that it can be very accurate for certain genera, reactions and metabolic systems whilst broad for others. Besides, the EpiPath cohort consists of only 115 participants which is a considerable number for a study on ELA, with the psychosocial stress test and with full immune and psychological profiling. Nonetheless, this sample size is considered small for a microbiome studies, where statistical screening leads to multiple testing, reducing the statistical power for detecting individual associations. Additionally, the glucose and cortisol measurements were only measured for a smaller set of participants (cortisol n=70, glucose n=42) diminishing our original sample size. Likewise, following metabolic modelling I was able to successfully model only 98 participants. Although Chapter 4 provides the mechanistic evidence for the observational evidence described in Chapter 2, the results from Chapter 3 remained observational and it was not possible to identify the direction of the interaction between the stress reaction and changes in the microbiome yet. In addition, as EpiPath is an adoption cohort, metadata such as the mode of birth, if they were ever collected, were never transferred to the adoptive parents. It is also possible that these data could be interpreted as the early inoculation with different microbiomes that simply persisted until 24 years later. The invasive nature of the ELA questions meant that compromise on microbiome-specific metadata, such as dietary habits and oral health status, was unavoidable if maximum participation in the study was to be ensured. Such information would have enhanced the mechanistic potential of this dataset. Information on particular diet habits could strengthen my simulations and guide me towards a more targeted approach (Dimitrov and Hoeng, 2013). Similarly, knowledge of potential oral complications such as carries or periodontitis would have allowed me to explore directly pathways known to be involved.

Nevertheless, I have for the first time clearly shown that the ELA has a long-term impact on the composition and metabolic capacity of the OM which uses microbial metabolites to interact with the host`s stress and immune responses. I have published and exposed observational and mechanistic evidence of this web of interactions that leads to the ELA phenotype. With all the aforementioned findings and strengths and despite the 16S sequencing limitations and lack of additional biological samples I have proven the microbial metabolites to control the see-saw of the ELA-OM contact. I have also demonstrated that oral samples collected from non-microbiome centered cohorts of diverse research fields can be used to explore the role of the oral microbiome in the context of each study.

5.5 Conclusion and perspective

Taken together all the findings from this thesis, I can describe the microbiome as a new "organ" or the human-microbiome as a "superorganism" (Baquero and Nombela, 2012, Salvucci, 2019). I can now confidently confirm the existence of an OM-Brain axis. A logical extension of my work would be the suggestion that OM and oral health should be investigated as an early detection screening ELA associated diseases. Further understanding of targeted pathways and mechanisms the OM involves for the host modulation are essential and could be optimised for intervention therapies eventually. This PhD journey on host-microbe interactions around ELA-OM has spawn many new hypotheses and ideas. Going back at the early life environment, how is it shaping the OM composition? What mechanisms are used to regulate the metabolic programming of the oral bacteria? Is this applied to other microbiomes like gut and lung? My data clearly demonstrate this interaction on this ELA model, however how often and under what context this interaction occurs? Is this only happenning during the development of the immune system in early life, or is this a constant life-long interaction signifying an active axis between the bone marrow and the OM, modifyied by ELA? In addition, further mechanistic-focus studies are essential to explore exactly which QS mechanisms the OM uses to communicate with the host and which bacterial metabolic assets explain this. Regarding the microbiome-immune system crosstalk, which signalling pathways are involved in the oral cavity for the mediation of these interactions and are these occurring locally or also distally? Last, is the microbiome the "saviour" or "driver" of disease?

With new biological questions, new scientific hypothesis rise and consequently there is the emerging need for prospective studies. My propositions and ideas for future study designs that would fill some gaps of this thesis and answer fresh questions are listed below:

5.6 Future study designs and outlook ideas

- Explore taxonomic associations with Th17 cells in the EpiPath
- Assess the impact of microbial metabolites on the stress-induced cortisol and glucose kinetics
- Experimental validation of the OM metabolome using our pipeline on a different cohort
- Validation of our findings on another ELA – institutionalisation cohort
- Follow-up studies using shotgun metagenomics to refine our reported associations down to species and strain level
- Follow-up studies with precise design and longer term investigations on ELA-microbiome-metabolome on diverse ELA models.
- Follow-up studies with microbial metabolites inducing epigenetic modifications as a mechanistic approach
- Reproduce my OM-Brain axis results on a similar cohort
- Compare Oral-Brain axis with Gut-Brain axis in a future ELA cohort

To close, it is clear that the OM has a bifacial relationship with ELA and the host`s physiological systems including immune system, HPA axis and metabolism. In context of health and disease, it remains unclear where the oral microbiome is sided. In the context of ELA and the seeding and establishment of the ELM it appears logical that this early interaction programming the microbial metabolome and shaping immune tolerance at the same time. Therefore, interest on consider it as an environmental mediator and moderator of ELA should be considered as next step. It is essential that future human studies keep a holistic approach in investigating the ELA-Oral-Brain axis and the prospective mechanistic pathways detected would require a targeted animal model in order to understand exactly what and how it happens at these first 1000 days.

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Appendix 1 – List of Publications

Charalambous EG, Mériaux SB, Guebels P, Muller CP, Leenen FAD, Elwenspoek MMC, Thiele I, Hertel J, Turner JD. Early-Life Adversity Leaves Its Imprint on the Oral Microbiome for More Than 20 Years and Is Associated with Long-Term Immune Changes. Int J Mol Sci. 2021 Nov 24;22(23):12682. doi: 10.3390/ijms222312682. PMID: 34884490; PMCID: PMC8657988.

Holuka C, Merz MP, Fernandes SB, **Charalambous EG**, Seal SV, Grova N, Turner JD. The COVID-19 Pandemic: Does Our Early Life Environment, Life Trajectory and Socioeconomic Status Determine Disease Susceptibility and Severity? Int J Mol Sci. 2020 Jul 19;21(14):5094. doi: 10.3390/ijms21145094. PMID: 32707661; PMCID: PMC7404093.

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EGC contributed 100% to the data generation, data analysis, data visualization, writing and final editing.

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Abstract: The early-life microbiome (ELM) interacts with the psychosocial environment, in particular during early-life adversity (ELA), defining life-long health trajectories. The ELM also plays a significant role in the maturation of the immune system. We hypothesised that, in this context, the resilience of the oral microbiomes, despite being composed of diverse and distinct communities, allows them to retain an imprint of the early environment. Using 16S amplicon sequencing on the EpiPath cohort, we demonstrate that ELA leaves an imprint on both the salivary and buccal oral microbiome 24 years after exposure to adversity. Furthermore, the changes in both communities were associated with increased activation, maturation, and senescence of both innate and adaptive immune cells, although the interaction was partly dependent on prior *herpesviridae* exposure and current smoking. Our data suggest the presence of multiple links between ELA, Immunosenescence, and cytotoxicity that occur through long-term changes in the microbiome.

Keywords: early-life adversity; early experience; microbiome; bacterial community; oral microbiome; developmental origins of health and disease; immune system; host-microbe interactions

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1. Introduction

Early-life adversity (ELA) is defined by a poor environment and conditions in early life that induce intense psychophysiological stress [1]. It is mostly observed together with low socioeconomic status and is pathophysiologically correlated with a lifelong imbalance of health and disease [2]. The first 1000 days from conception to 2 years is the most vulnerable life period [3]. At birth, the body is almost fully formed; however, many biological systems continue to mature over the following years. Research on the lifelong health and disease balance has shown the significance of the environment during this period on multiple disease phenotypes [4], including cardiovascular, allergic, and autoimmune disorders, as well as mental disorders [5–17]. There has been a focus on the molecular mechanisms and the cellular phenotype behind the effect of stress and adversity on immune and endocrine systems as well as epigenetic modifications [5,6,18,19].

ELA has been reported to influence health trajectories via the immune system [18,20,21], with a clear ELA-associated immunophenotype centred around the activation and functional status of T lymphocytes. In the institutionalisation model of early-life stress, strong T-cell immunosenescence has been reported [18,21,22]. Immunosenescence is a form of accelerated immune ageing. The CD57 T- and NK- cell immunosenescence marker is absent in early life and increases with age, with high numbers of such cells in the elderly population. Immunosenescence is driven by chronic inflammation or recurrent viral infections such as CMV [23]. NK functionality is also highly impacted by recurrent reactivation of CMV inducing NK cell exhaustion, increased cytotoxicity, and senescence [24]. Additionally, such viral infections potentially program the immune system [21,22]. Latent CMV infection of haematopoietic progenitor cells reduces GR transcription and translation, impacting immune cell maturation, which can be dependent on CMV reactivation [19,25,26].

The environment is the most critical factor shaping ELA. In the immediate postnatal period, the immune system starts maturing and the first body-area-specific microbial communities are established. Once established, the microbiome modulates the host [27], a mechanism to protect symbiotic microbial communities, where cases of microbial dysbiosis can be fatal [28,29]. The ELM plays important roles in an infant's subsequent development [5,6] and a long-term health trajectory [5–11]. Nevertheless, the development of the ELM is critically impacted by the environment, mode of birth, early-life nutrition, and environmental exposure, which leave a clear lifelong trace [16,30]. For example, maternal milk is rich in essential nutrients, protective antibodies, and components essential for the developing microbiome, such as human milk oligosaccharides (HMOs) and short-chain fatty acids (SCFAs) [31–36].

The oral microbiome (OM) is composed of various distinct, smaller communities within the oral cavity [37–41] that are robust, stable, and particularly resilient [42,43], particularly to antibiotic therapy [43–47]. Moreover, saliva contains actively secreted components such as cortisol, glucose, lactate, urea, and proteins, such as polypeptides, glycoproteins (cystatins, mucins, and immunoglobulins) and antimicrobial peptides (histatins, defensins, and immunoglobulins-IgA). Many of these are energy sources for the OM, and salivary glycoproteins are the principal nutrient source. These substrates are crucial for the development of multispecies communities and their preservation [41,46,48], and enhance the resistance of the community to environmental stressors [41,49,50]. The long-term stability of the OM leads to the hypothesis that, once established in early life, it remains stable, robust, and resilient, retaining an imprint of the early environment [42,43].

We previously reported higher virally mediated activation and senescence of the immune system in association with ELA in the EpiPath cohort [22,51]. This cohort consists of young adults exposed to ELA by either institutionalisation or separation from their parents at birth, and were subsequently adopted into Luxembourg, while controls were raised by their biological parents. With the growing evidence of a microbiome–immune–system interaction, we attempted to identify if institutionalisation left a mark on the OM of the adoptees. In this study, we sequenced the 16s-rRNA from the buccal and salivary bacterial communities from our cohort. Integrating this with the full immunophenotype, we identified associations with various taxa and analysed how the microbiome interacts with the immune system.

2. Results

The V4 region of the bacterial 16S gene was successfully amplified from both buccal swabs and salimetrics oral swabs for the 115 members of the EpiPath cohort, and a total of 45 Gbp sequencing data were obtained. All samples were successfully processed using mothur and a total of 288 and 371 genera from 24 phyla were identified for buccal and salivary samples respectively. The saliva and buccal swabs from the EpiPath cohort were examined independently as they are two distinct oral communities from the same participants.

2.1. Microbial Diversity and Overall Microbial Composition

2.1.1. Salivary Microbiome (α- and β-Diversity)

We identified sequences from all of the 24 principal bacterial phyla in the overall salivary microbial community. Within these 24 phyla, the most abundant in both control and ELA groups were *Actinobacteria*, *Proteobacteria*, *Firmicutes*, and *Fusobacteriota* (Figure 1A). The most abundant genera of the salivary composition were *Acinetobacter*, *Micrococcaceae*, *Actinobacillus*, *Rothia*, *Corynebacterium*, *Micrococcus*, *Actinomyces*, *Alloprevotela*, *Porphymonas*, *Fusobacterium*, *Weeksellaceae*, *Flavobacterium*, *Bradyrhizobium*, *Porphyromonas*, *_ Comamonas*, *Olsenella*, *Fluviicola*, *Fusobacterium*, *Absconditabacteriales _(SR1)_ge*, *Streptobacillus*, *Fretibacterium*, *JGI_0000069-P22_ge*, *Capnocytophaga*, *Pseudarcicella*, *Tannerella*, *Prevotella*, and *Campylobacter* (Figure 1B). There was no difference in α-diversity between the controls and the adoptees in terms of diversity (controls: mean = 13.87872, SD = 7.127721, adoptees: mean = 14.14869, SD = 6.601899, Wilcoxon rank sum test $p = 0.7579$) and evenness (controls: mean = 0.5619667, SD = 0.06864806, adoptees: mean = 0.5646776, SD = 0.06172601, Wilcoxon rank sum test $p = 0.9461$). Plotting the Shannon evenness index against the inverse Simpson diversity index confirmed that there analogous diversity and evenness between the controls and adoptees (Supplementary Figure S1). Principal coordinate analysis could not detect systematic differences either (Supplementary Figure S1).

2.1.2. Buccal Microbiome (α- and β-Diversity)

As for the salivary microbiome, we identified sequences from all the 24 principal bacterial phyla in the buccal microbial community. The most abundant phyla were the same as in the salivary microbiome: *Actinobacteria*, *Proteobacteria*, *Firmicutes*, and *Fusobacteriota* (Figure 2A). The most abundant genera were *Actinomyces*, *Corynebacterium*, *Micrococcaceae*, *Rothia*, *Alloprevotela*, *Porphymonas*, *Fusobacterium*, *Actinobacillus*, *Bradyrhizobium*, *Haemophilus*, *Methylobacterium-Methylorubrum*, *Oxalobacteracea*, *Actinomyces*, *Neisseria*, *Paucibacter*, *Lautropia Cardiobacterium*, *Brucella*, *Alysiella*, and *Campylobacter*, therefore revealing a substantial overlap in the detected genera between the saliva and the buccal microbiome (Figure 2B).

As for the salivary microbiome, we observed a very similar diversity and evenness between controls and adoptees as measured by the inverse Simpson index (controls: mean = 12.85828, SD = 10.117292, adoptees: mean = 14.72315, SD = 8.991065, Wilcoxon rank sum test $p = 0.08803$). The Shannon evenness index was similar between adoptees and controls, and, in both, it was higher than in the salivary microbiome (controls: mean = 0.5137724, SD = 0.10141122, adoptees: mean = 0.465861, SD = 0.08482336, Wilcoxon rank sum test $p = 0.09024$). α-diversity was again similar in the controls and the adoptees in terms of evenness (Wilcoxon rank sum test $p = 0.08803$) and diversity (Wilcoxon rank sum test $p = 0.09024$). Plotting the Shannon evenness index against the inverse Simpson diversity index revealed no systematic differences in diversity or evenness between the controls and adoptees (Supplementary Figure S1). Similarly, principal coordinate analysis indicated no differences between adoptees and control (Supplementary Figure S1).

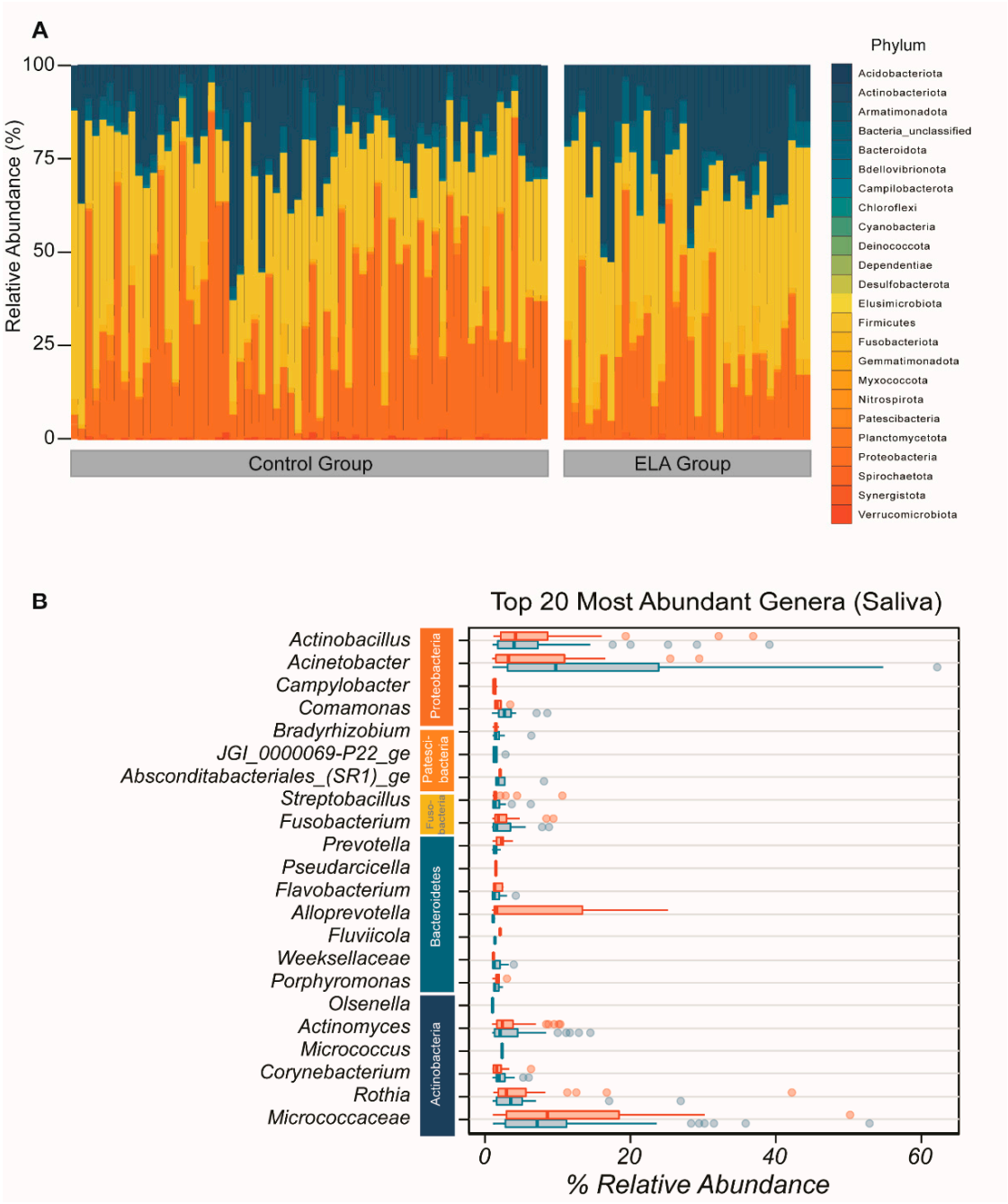

Figure 1. Overall composition of salivary bacterial community. (**A**) Overall microbial composition displayed in stacked area bar plot with the percentage relative abundance of all phyla found in each participant in both study arms. (**B**) Top 20 most abundant genera by mean abundance arranged graphically by phyla. Vertical line = mean; rectangle = 1st to 3rd quartile; horizontal lines = 2.5th to 97.5th percentile. Outliers are indicated as individual data points. Blue, control group; red, ELA group.

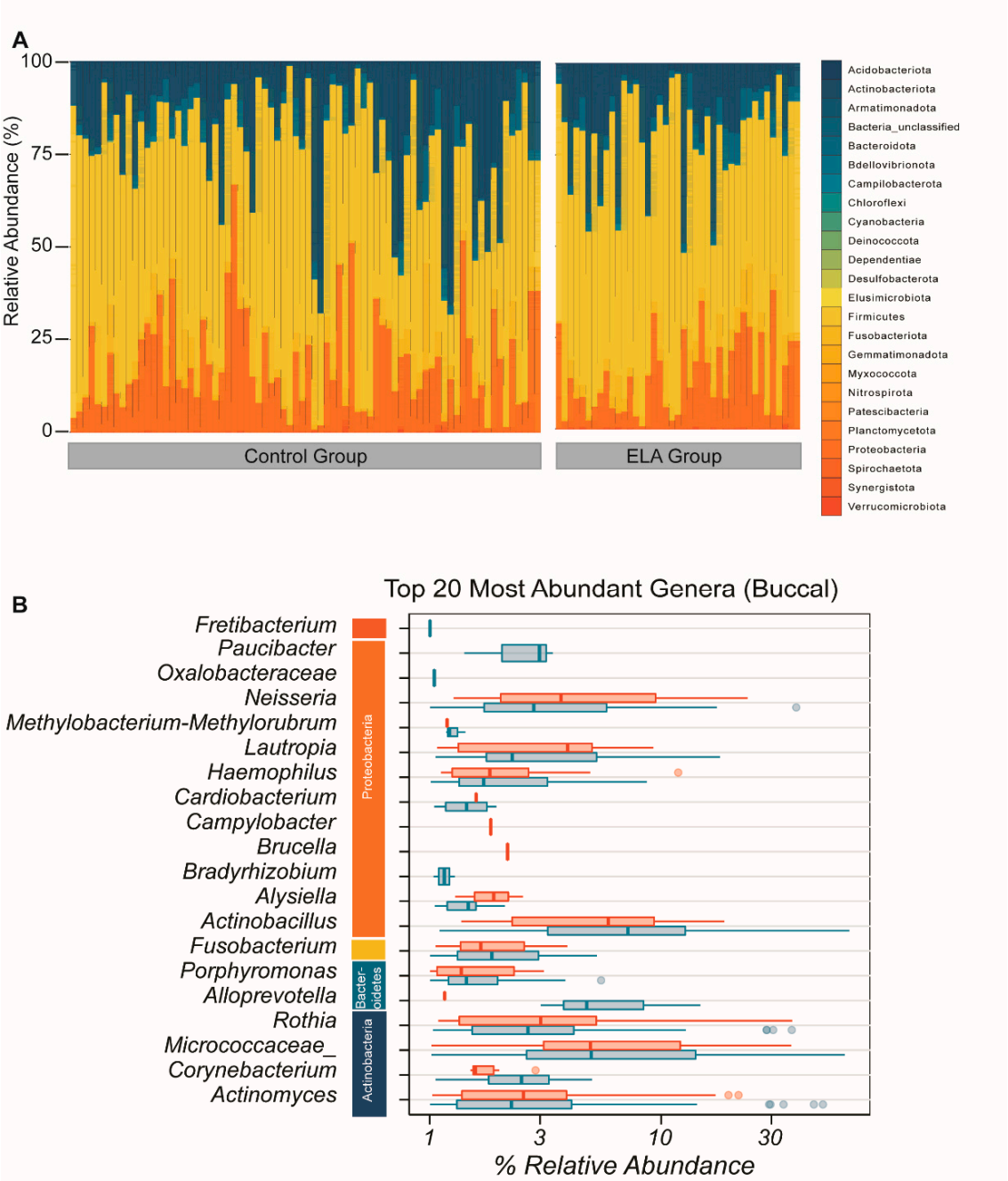

Figure 2. Overall composition of the buccal bacterial community. (**A**) Overall microbial composition displayed in stacked area bar plot with the percentage relative abundance of all phyla found for each participant in both study arms. (**B**) Top 20 most abundant genera by mean abundance arranged graphically by phyla. Vertical line = mean; rectangle = 1st to 3rd quartile; horizontal lines = 2.5th to 97.5the percentile. Outliers are indicated as individual data points. Blue, control group; red, ELA group.

2.1.3. Salivary and Buccal Microbiomes Are Two Separate Entities

To ensure that sample collection was performed correctly and that we had two distinct communities, we compared the diversity and evenness of the salivary and buccal samples. We found a low correlation between the salivary and buccal communities in both the inverse Simpson diversity index (Figure 3A, $p = 0.47$, rho = −0.07372058, Spearman's rank correlation test) and Shannon evenness index (Figure 3B, $p = 0.8759$, rho = 0.01595802, Spearman's rank correlation test), giving evidence in favour of the hypothesis that, despite their close physical proximity, they can be seen as distinct communities. Comparing the

salivary and buccal microbiomes by group, the diversity ratios of the salivary against buccal communities were similar between the controls and adoptees (Figure 3A, controls: $p = 0.3311$, rho = −0.1222465; adoptees: $p = 0.7898$, rho = 0.04812834; Spearman's rank correlation test, Figure 3B; controls: $p = 0.9578$, rho = 0.009692513; adoptees: $p = 0.7898$, rho = −0.04812834; Spearman's rank correlation test). This suggests that the overall composition between the controls and adoptees may be similar, but differences would be seen at the phyla level.

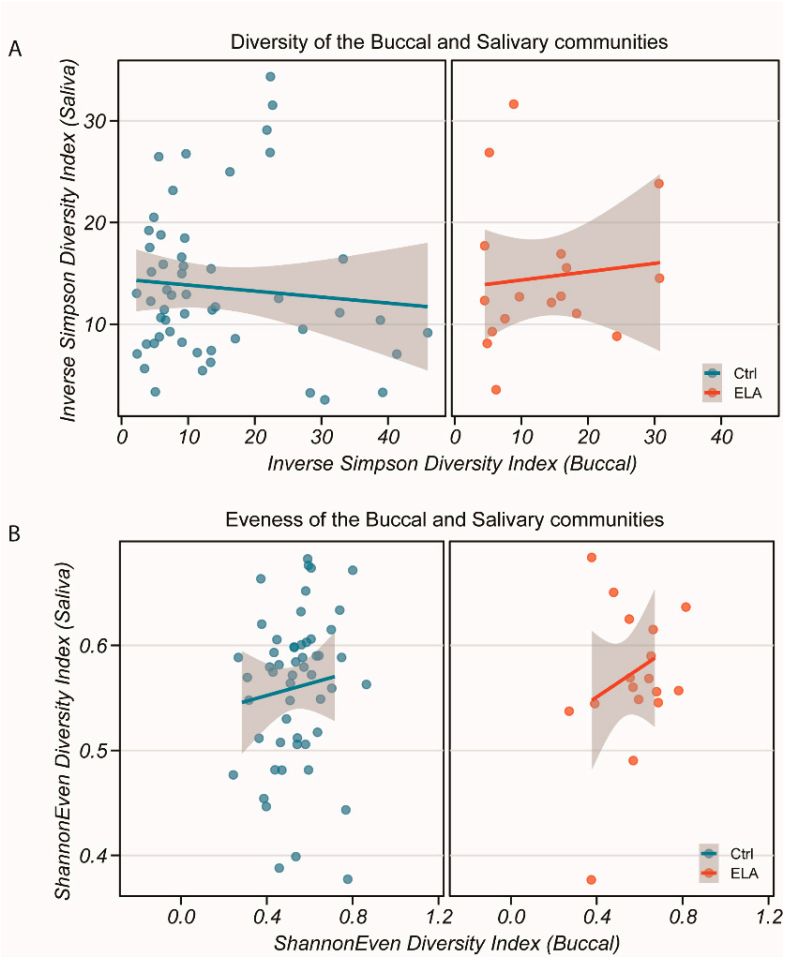

Figure 3. Diversity and evenness of the salivary and buccal bacterial communities in both study groups. (**A**) Inverse Simpson diversity index of saliva against buccal communities for control (**left**) and ELA (**right**). (**B**) Shannon evenness index of saliva against buccal communities for control (**left**) and ELA (**right**). No correlation was found between either community for either measure or group (Spearman's rank correlation test, $p > 0.47$). Grey shaded area: 95% confidence interval.

2.2. ELA Induces Differences in Specific Taxa in Both Salivary and Buccal Communities

Investigating the abundance levels of phyla and genera highlighted differences in the community composition across the ELA group and healthy controls (Table 1). While there were no differences in the phyla level in the buccal data, *Proteobacteria* and *Verrucomicrobiota* were significantly lower (FDR < 0.05 for both) in the adoptees in comparison to the controls (Supplementary Table S1A, Figure 4A,B) in the saliva microbiome as detected in fractional regression analyses. Analyses of deeper taxonomy revealed two of the most abundant genera of the *Proteobacteria* phylum, *Comamonas* and *Acinetobacter*, to be significantly lower in the saliva of adoptees compared to controls alongside *Aquabacterium* and unclassified *Comamonadaceae* (Table S1B, Figure 4C–F). In conclusion, while we could not detect systematic differences in the buccal microbiome between the ELA group and the controls, the

saliva microbiome was structurally different in its composition, with a prominent role for *Proteobacteria* genera (Figure 4A).

Table 1. An overview of the bacterial taxa associated with all the tested covariates in both saliva and buccal microbiomes.

	Saliva			Buccal		
	Number of Associations (FDR < 0.05)	**Positively Associated Taxa**	**Negatively Associated Taxa**	**Number of Associations (FDR < 0.05)**	**Positively Associated Taxa**	**Negatively Associated Taxa**
Smoking	0	-	-	5	-	*Pasteurellaceae* (unclassified), *Neisseria*, *Capnocytophaga*, *Neisseriaceae* (unc [1]), *Planococcaceae* (unc)
ELA vs. control	4	-	*Aquabacterium*, *Comamonas*, *Comamonadaceae* (unc), *Acinetobacter*	0	-	-
Anti-CMV seropositive	2	-	*Pseudomonas*, *Oxalobaceraceae* (unc)	9	*Alysiella*, *Neisseria*	*Sphingomonas*, *Acinetobacter*, *Oxalobacteraceae* (unc), *Bradyrhizobium*, *Flavobacterium*, *Methylorubrum*, *Comamonadaceae* (unc)
Anti-EBV seropositive	0	-	-	1	*Neisseria*	-
HSV	0	-	-	0	-	-
CD4+ CD57+	2	*Selenomonas*	*Oxalobaceraceae* (unc)	4	*Selenomonas*, *Capnocytophaga*, *Campylobacter*, *Lautropia*	-
CD8+ CD57+	0	-	-	0	-	-
Total CTLs	0	-	-	0	-	-
Total T_h cells	0	-	-	0	-	-

[1] unc = unclassified.

2.3. Environmental Covariates

Next, we investigated the impact of environmental factors on the OM to potentially explain the effects of ELA described above (Table 1).

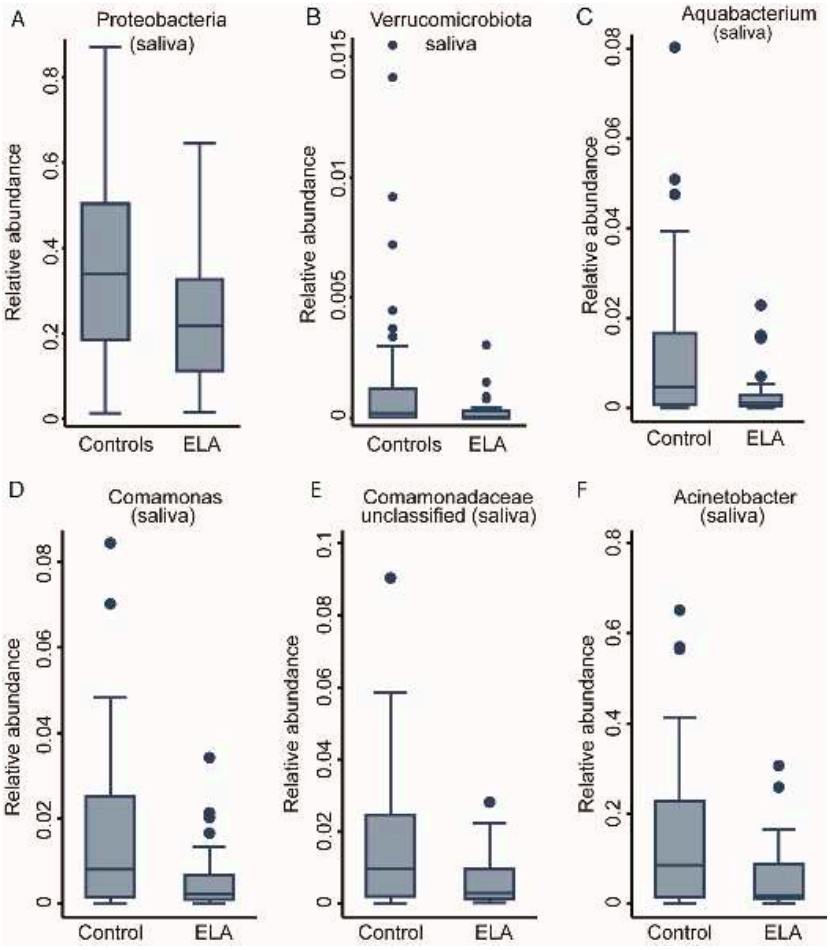

Figure 4. Taxonomic differences between study groups in the salivary bacterial community. Box plots of two phyla, (**A**) Proteobacteria and (**B**) Verruomicrobiota, as well as four genera: (**C**) Aquabacterium, (**D**) Comamonas, (**E**) Comamonadaceae, unclassified and (**F**) Acinetobacter. All are significantly associated with study group; fractional regressions against study group were calculated to determine significance (FDR < 0.05). Horizontal line = mean; rectangle = 1st to 3rd quartile; vertical lines = 2.5th to 97.5th percentile. Outliers are indicated as individual data points.

2.3.1. Smoking

As lifestyle has a pivotal role in the development of the microbiome, we assessed the effect of smoking on the OM by including smoking status (binary: smokers vs. non-smokers) into the regression modelling (Table 1). No significant genera were detected for the salivary community, whereas from the buccal community, we exposed five genera negatively associated with smoking: three from the *Proteobacteria* phylum, *Neisseria*, *Neisseriaceae_unclassified*, and *Pasteurellaceae_unclassified*; 1 from the *Bacteroidetes* phylum and *Capnocytophaga* genus; as well as one from the *Firmicutes* phylum and *Planococcaceae_unclassified* genus (FDR < 0.05, Figure 5A–E). In sensitivity analyses, we removed smoking as a covariate from the regression equations for the FDR-corrected significant genera to explore potential effect mediation through smoking, but the results remained virtually unchanged. The full results for the buccal and saliva microbiomes can be found in Supplementary Table S2.

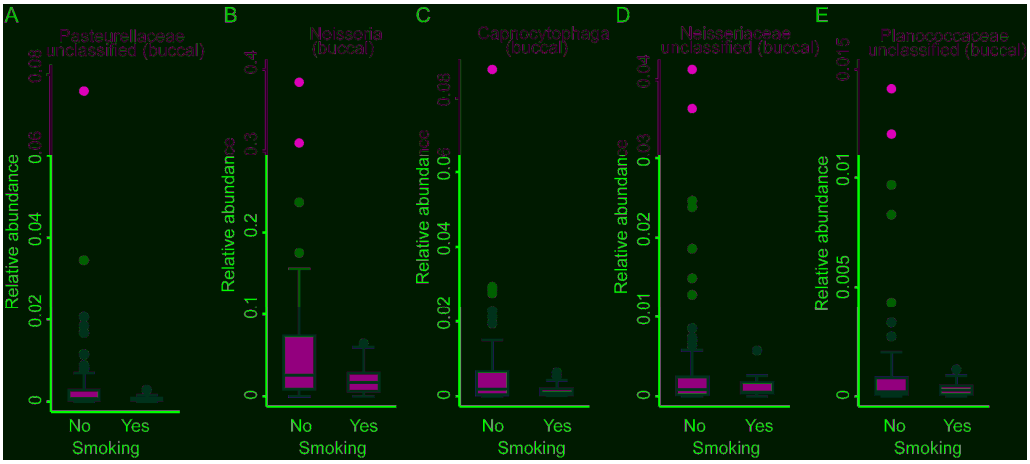

Figure 5. Taxonomic differences in the buccal bacterial community associated with smoking. Box plots of five genera: (**A**) Pasteurellaceae unclassified, (**B**) Neisseria, (**C**) Capnocytophaga, (**D**) Neisseriaceae unclassified, and (**E**) Planococcaceae unclassified. All are significantly associated with study group; fractional regressions against smoking were calculated to determine significance (FDR < 0.05). Horizontal line = mean; rectangle = 1st to 3rd quartile; vertical lines = 2.5th to 97.5th percentile. Outliers are indicated as individual data points.

2.3.2. Prior Viral Infections

We previously reported that viral infections may mediate the early-life immunophenotype [22]. Consequently, we tested whether prior viral infection, measured as anti- HSV, EBV, and CMV seropositivity, affected the oral bacterial communities. We achieved this via fractional regressions with the antibody titre (binary: positive vs. negative) as the predictor of interest and the genus abundance as the response variable (Table 1). Due to low case numbers of positive titre results for CMV, we could not adjust for basic covariates without inducing numerical instability in the fitting procedure. While HSV titres did not show any association, a positive EBV titre was positively associated with the abundance of the *Neisseria* genus in the buccal microbiome (FDR < 0.05, Figure 6A). However, anti-CMV antibody titres showed a very strong association with the OM. In total, 10 genera had a significant association with CMV titres. Nine genera from the Buccal community, eight derived from the *Proteobacteria* phylum (*Acinetobacter*, *Bradyrhizobium*, *Comamonadaceae_unclassified*, *Methylobacterium-Methylorubrum*, *Oxalobacteraceae_unclassified*, and *Sphingomonas* genera) unveiled a negative association, whereas the genera of *Alysiella* and *Neisseria* demonstrated a positive association. One genus from *Bacteroidetes* phylum, *Flavobacterium*, also appeared to be negatively associated with positive CMV titres (FDR < 0.05, Figure 6B–J). Two genera of the Salivary community from the *Proteobacteria* phylum, Pseudomonas and *Oxalobacteraceae_unclassified*, exhibited a negative association (FDR < 0.05, Figure 7A,B). The full results for the buccal and the saliva microbiome antibody titre associations can be found in Supplementary Table S3. In a further step of sensitivity analysis, we included a positive antibody titre as a covariate into the regression models to investigate the differences in the genus abundances between ELA and controls. However, the results virtually remained the same, indicating either insufficient statistical power to detect potential mediation or that CMV exposure does not mediate ELA-related changes in the OM. This suggests that, unlike increased immunosenescence, the changes we saw in the oral bacterial community are independent of prior exposure to Herpesviridae.

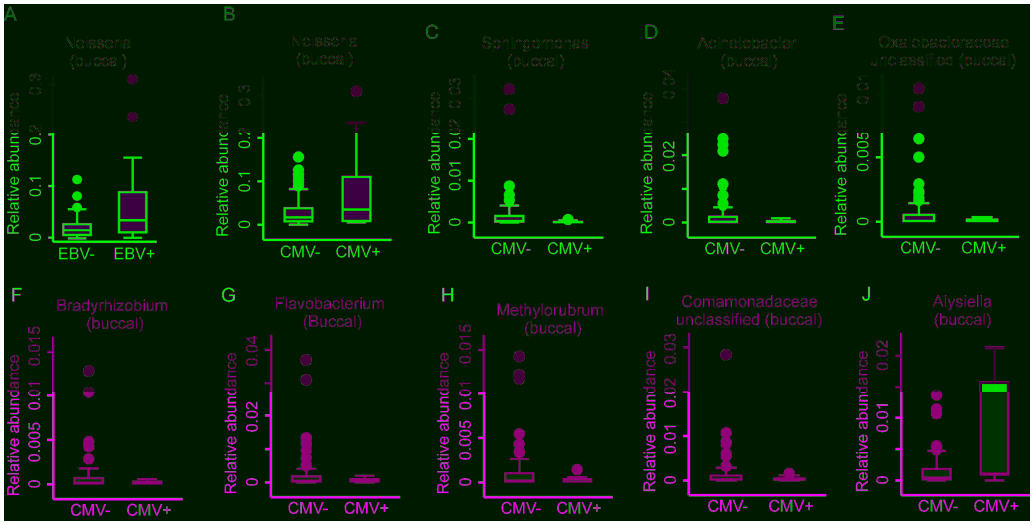

Figure 6. Taxonomic differences in the buccal bacterial community associated with anti-herpesviridiae serological status. Box plots of one genus (**A**) *Neisseria* significantly associated with anti-EBV antibody titres. Nine genera, (**B**) *Neisseria*, (**C**) *Sphingomonas*, (**D**) *Acinetobacter*, (**E**) *Oxalobacteraceae unclassified*, (**F**) *Bradyrhizobium*, (**G**) *Flavobacterium*, (**H**) *Methylorubrum*, (**I**) *Comamonadeceae unclassified*, and (**J**) *Alysiella*, were significantly associated with anti-CMV antibody titres. Fractional regressions against the presence of anti-EBV and anti-CMV antibodies were calculated to determine significance (FDR < 0.05). Horizontal line = mean; rectangle = 1st to 3rd quartile; vertical lines = 2.5th to 97.5th percentile. Outliers are indicated as individual data points.

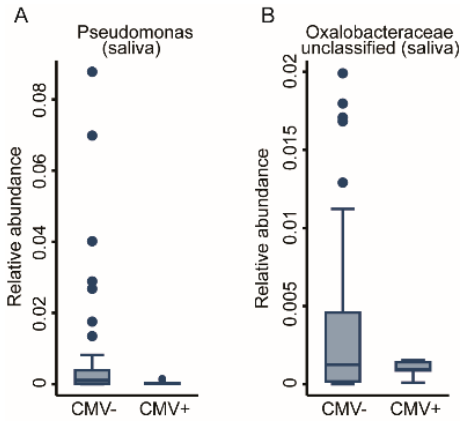

Figure 7. Taxonomic differences in the salivary bacterial community associated with anti-CMV serological status. Box plots of two genera, (**A**) *Pseudomonas* and (**B**) *Oxalobacteraceae unclassified*, which were significantly associated with CMV antibody titres. Fractional regressions against the presence of anti-CMV antibodies were calculated to determined significance (FDR < 0.05). Horizontal line = mean; rectangle = 1st to 3rd quartile; vertical lines = 2.5th to 97.5th percentile. Outliers are indicated as individual data points.

2.4. Fractional Regression Models of the Immune–Microbiome Interactions

In the next step, we fitted a series of fractional regression models integrating the relative abundance of the taxonomic levels in the salivary and buccal compositions with our previously published immune-system profiling. Among the full dataset of 48 immune cell populations, we identified 11 significant associations with genera, most importantly for T cells and NK cells (Table 1).

2.4.1. Association with CD4 T-Cell Immunosenescence

Immunosenescence is a common result of adversity. Thus, we decided to look for possible associations between adversity, microbiome, and accelerated ageing of immune

cells (Table 1). For screening the OM associations with the share of CD57 -positive CD4 and CD8 cells, we used multivariable fractional regressions including the genus abundance as the response variable, and the share of CD57-positive CD4 and CD8 cells as a predictor of interest and the basic set of covariates. Additionally, we included the study-group variable as a covariate to control for potential confounding factors related to ELA status. CD8 T cells were previously reported to be significantly associated with CMV [22], but we found no associated taxonomic markers from the OM. From CD4 T-cells tests, we identified six strong taxonomic associations. Two genera from the salivary microbiome, *Selenomonas* from the *Firmicutes* phylum showed a positive association and *Oxalobacteraceae_unclassified* from the *Proteobacteria* phylum showed a negative association. Four genera from the buccal community: *Selenomonas* from *Firmicutes*, *Capnocytophaga* from the *Bacteroidetes* phylum, and *Campylobacter* and *Lautropia* from the *Proteobacteria* phylum, displayed a positive association (FDR < 0.05, Figure 8A–F). For further exploration, we fit additional fractional regressions using the number of T-helper cells and T-killer cells as predictors of interest using the same set of covariates as before, finding no additionally significant associations after correction for multiple testing. Summary statistics for the buccal and saliva microbiomes are given in Supplementary Table S4.

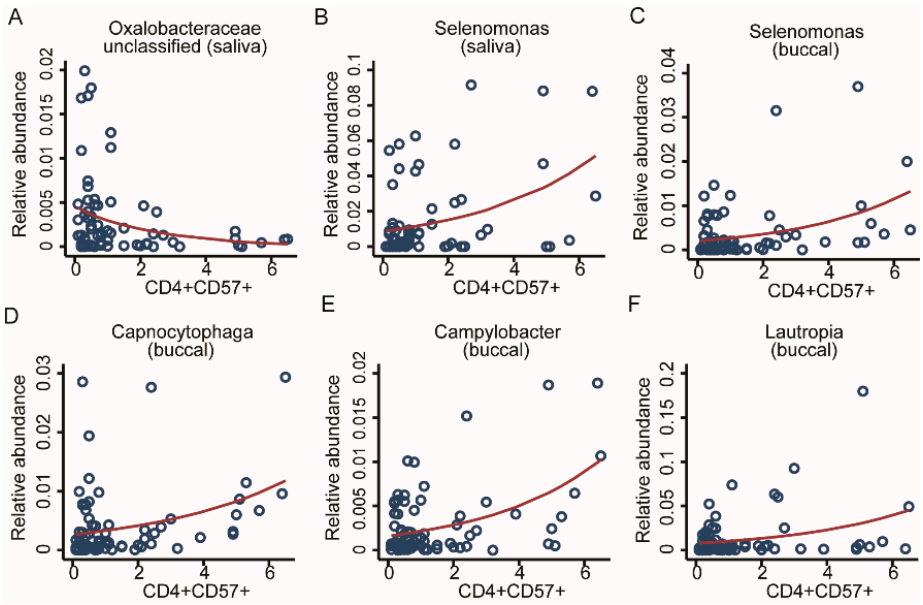

Figure 8. Taxonomic associations in both communities with immunosenescence. Scatter plots with regression lines for six genera. From the salivary community, two genera, (**A**) *Oxalobacteraceae unclassified* and (**B**) *Selenomonas*, as well as four genera from the buccal community, (**C**) *Selenomonas*, (**D**) *Capnocytophaga*, (**E**) *Campylobacter*, and (**F**) *Lautropia*, were all significantly associated with CD4 CD57 cell counts. Fractional regressions against CD4 CD57 cell counts were calculated to determine significance (FDR < 0.05). Regression lines were derived from fractional regressions with logistic parametrisation of the conditional mean.

2.4.2. Association with NK Cell Activity

Innate immune cells such as natural killer (NK) cells are the first line of defence and often interact with commensal bacteria. Adoptees of this cohort showed increased cytotoxicity on their NK cells [52]; hence, we thought to assess for a potential link with the microbiome. Through screening the OM for associations with various types of NK cells, we found three genera associated with cell counts with an FDR < 0.05, while seven additional associations reached an FDR < 0.1 (Supplementary Table S4), hinting that a better-powered study may find a broader association pattern. In the buccal community, the *Oribacterium* genus showed a negative association with the total number of NK cells and the total number of mid-maturation NK cells (FDR < 0.05, Figure 9A,B). In parallel, within the

salivary community, several genera were significantly associated with different stages of NK maturation. *Pseudomonas* was found to be positively associated with the total number of CD25 expressing NK cells, which reflects an association with the global activation of NK cells (FDR < 0.05, Figure 9C). The abundance of *Alloprevotella* was positively associated with the abundance of activated immature CD25CD56^{hi} expressing NK cells (FDR < 0.05, Figure 9D).

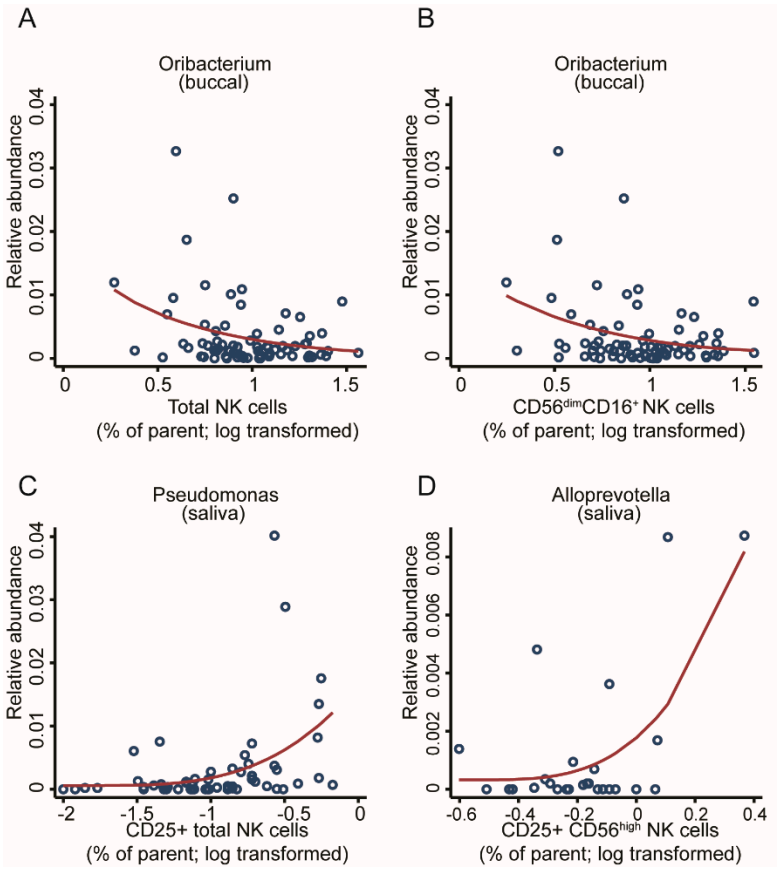

Figure 9. Taxonomic associations in both communities with natural killer cell activity. Scatter plots with regression lines for one genus from the buccal community, *Oribacterium*, was significantly associated with (**A**) the total number of NK cells and (**B**) the total number of mid-maturation NK cells as well as two genera from the salivary community: (**C**) *Pseudomonas*, associated with CD25^{+} NK cell counts; and (**D**) *Alloprevotella*, associated with CD25CD56^{hi} NK cell counts. Fractional regressions against NK cell counts were calculated to determine significance (FDR < 0.05). Regression lines were derived from fractional regressions with logistic parametrisation of the conditional mean.

Summary statistics for the buccal and saliva microbiome are given in Supplementary Table S4 and bacterial taxa are highlighted in Table 1.

3. Discussion

In this study, we identified taxonomic differences in the OM 24 years after adversity that were common throughout a cohort of diverse cultural and ethnic origins. We identified genera that had a significantly reduced abundance in the adoptees, which were significantly associated with smoking; immunosenescence of CD4 T cells; circulating number and activation status of NK cells; and anti-CMV and, to a lesser degree, anti-EBV titres. Importantly, we were able to see these differences in both the salivary and buccal microbiomes, both of which are readily accessible and both are regularly and easily sampled, even if the buccal microbiome is somewhat underexplored to date. Our data highlight the distinctness of the salivary and buccal microbiomes in distinct oral niches with unique microbial signatures.

Our findings from the EpiPath cohort closely mirror those of Reid et al. [53], although in significantly different microbial communities. We report differences in the abundance of taxa associated with early institutionalisation and CMV seropositivity. Considering that the gut microbiome (GM) is far more labile to lifestyle and environmental impact than the OM [53], our findings build upon those of Ried et al., opening the possibility of much longer-term studies, as the enhanced stability of the OM suggests that differences may be stable over many decades [53]. Expanding our analyses to associations with the immunosenescent CD4 T cells and the activation status of circulating NK cells strengthens the possible role of microbe–immune cross-talk in ELA and the potentially detrimental outcomes. Furthermore, at the family taxonomic level, we observed highly similar differences to those reported by Reid et al. (e.g., *Prevotella* vs. *Alloprevotella*, both from the *Prevotellaceae* family). This highlights the link between the oral and GMs, as numerous studies provide evidence of bacteria migrating from the oral cavity and colonising the gut, whereas there is no evidence of the opposite happening [54–57].

Our current findings show that institutionalised, genetically unrelated individuals share particular taxa, identifiable 24 years later, independent of the event of adoption. The buccal community, in contrast to the salivary community, appears to be more prone to lifestyle habits such as smoking, agreeing with previous reports that the salivary community remains stable despite lifestyle-hygiene-related mediations such as flossing [42,43,58]. This agrees with several prior reports of the stability and resilience of oral communities over time [39,41,43,46,47,59–62]. Although host genetics help to shape microbial communities, previous reports of low variance between twins suggest that the shared early environment is the key determinant of the long-term composition [43,60,62]. Longitudinal observations of twins revealed that the salivary microbiome has a stable core community at the genus level, and as twin lives diverge over time, environmental differences increase the diversity between the microbiomes of twins [60,61]. Furthermore, genetically unrelated people with a shared environment show similar environment-related effects on microbiome composition in the mouth as well as other communities [43,47,61]. Cohabitation appears to have a greater impact on the skin microbiome rather than gut and oral communities, persisting after the cohabitation is terminated [43,58,60,63], an effect that is thought to persist for the long term despite leaving or changing household [41,48,49].

The importance of the OM should not be underestimated. As for the GM, there is a direct interaction between the microbiome and both oral and systemic health. Multiple oral inflammatory microbiome-associated conditions such as periodontitis and carries have strong epidemiological and mechanistic associations to other systemic and gastrointestinal diseases [61,64]. Further associations over the years have identified oral marker links to systemic complications, including cardiovascular, immune, metabolic, respiratory, osteopathic, obstetric, and perinatal complications [64–66]. In both healthy and inflammatory statuses, viable oral bacteria are often found to travel from the mouth to the gut and are capable of achieving successful colonisation [54–56]. Schmidt et al. found that more than half of identified species often found residing in both mouth and gut exhibited signs of oral–gut transmission for all their study participants. Nearly one-third of these are taxa known to be highly dominant in oral communities [54,57,67]. Interestingly, this is a one-way observation: although oral strains can travel to and colonise the gut, the opposite is unlikely to occur [54,57]. Hence, as dental health research has been suggesting for years, oral microbial composition hinges on oral and dental health. In contrast to the prevailing GM, OM shows rising importance as an indicator of systemic health.

Although observational, we report numerous clear associations and correlations in our statistical model that demonstrate the crosstalk between the microbiome and the immune system. Microbial transmission across the gastrointestinal tract, direct microbial contact with tissue-resident innate cells, probable oral bacterial infection, circulating bacterial toxins, and molecular mimicry are all valid candidate pathways that may explain the observed relationship [65,66]. The ELM plays a crucial role in educating immune cells (immune tolerance) that are completely naïve at birth. As immune cells learn to recognise

host cells, they are also programmed to recognise antigens from the developing beneficial endogenous microbiome [68]. Tissue-resident dendritic cells harvest microbial antigens from local microbial communities and present them to other immune cells [68]. In germ-free mice, the absence of a microbiome during the early-life period alters immune functions and induces structural defects in lymphoid tissues. In the presence of microbial communities, these tissue structures form normally. Despite many such observations, it is unclear how this acts mechanistically to alter the formation of epithelial barriers. Evidence from the gut suggests that bacteria can direct the glycosylation of luminally exposed surface proteins, a process whose outcome differs in germ-free mice [69–71]. Initially, T_h17 cells are absent in germ-free mice and only appear upon microbial colonisation [68,69].

It is now well-established that the relationship between stress and chronic disease starts in utero, as susceptibility and occurrence of disease can be predefined by maternal stress [72]. During this period, the naïve, uneducated, immune system develops [73]. NK cells are part of the body's first line of immune defence, interacting with other immune cells as well as pathogens. In the majority of chronic diseases associated with the early-life environment, NK cells appear to either have an impaired function or an exaggerated cytotoxic activity [74,75]. The most studied NK cell populations are the CD56brightCD16$^-$ and CD56dimCD16bright cells and the associated cytotoxic CD56dim and cytokine-producing CD56bright cells [76]. NK cell cytotoxicity is initiated by target cell contact and recognition, which leads to immune synapse formation, resulting in NK-cell-induced target-cell death. The proliferation and expansion of NK cells depend on CD4$^+$ T_h1 cells. Nevertheless, due to the bidirectional relationship between innate and adaptive immunity, NK cells impact CD4$^+$ and CD8$^+$ T cells through cytokine production [77]. In the absence of short-chain fatty acids (SCFAs), metabolites produced from fibre fermentation by the local microbiome communities, certain CD4 T-cell subsets do not differentiate. Furthermore, naïve CD8 T cells do not differentiate into memory cells in germ-free mice [68,78,79]. The activation of NK cells by pathogen-associated molecular patterns (PAMPs) may initiate an unwanted response in the microbiome and lead to a strong inflammatory response [80]. Similarly, pathogen-driven activation of NK cells can result in increased on-site cytotoxicity, which can also be harmful to local microbial communities. Correspondingly, microbiome members regulate homeostasis by inducing NK cell expansion and cytokine production or driving the proliferation of anti-inflammatory cytokine-producing NK cells, a common event observed with tissue-resident cells and microbiome crosstalk [81]. Furthermore, immunomodulatory properties of the bacterial community may drive antiviral defences regulating the outcome of viral infection [82].

The OM is intimately linked to oral health. Poor oral health is often approached in an eco-social framework, as it is known to be associated with psychosocial adversity [83]. Both epigenetic and behavioural pathways were linked to poor oral health [83]. One of the most studied causal routes is diet. Affordability and access to a nourishing diet" are strongly influenced by socio-economic status [83], which in turn is linked to the composition of the OM. Detrimental shifts in the microbial composition associated with poor immune responses and mental health were documented for both hospitalised and long-term care home residents [84]. The multidirectional interconnected relationship between the microbial composition, the host's immunological status, and the resulting life-long health trajectory is most probably highly dependent on constant exposure to particular irritants [84].

Our observation that psychosocial adversity is associated with changes in the OM opens many possibilities for future research. The collection of oral samples, primarily saliva, has been the sampling media of choice for psychobiology, lifestyle, and other social to clinical research areas for many decades. Saliva has long been recognised as an accurate, noninvasive, and cost-effective diagnostic approach that can be tailored to personalised medicine strategies [65,85]. Here, we opened up the possibility of using standard salivary swabs previously collected for microbiome studies. Such studies have the potential to provide a more holistic view of host–microbe interactions and the role of the microbiome in

health, which is a potential that can now be applied in nearly all areas of psychobiology (and further afield). Our data also provide preliminary mechanistic insights and the perspectives for future detailed mechanistic studies. We know that early oral microbial colonisation is associated with IL-17-producing cells [86], and subsequent chronic oral disease is often initiated by T_h17 cells and IL-17 [87–89]. In our EpiPath cohort, there was a strong ELA-associated increase in immunosenescence-associated chronic inflammation, together with increased T_h17 cell numbers, although this narrowly missed significance ($p = 0.06$, [51]). The ELA-associated immunophenotype is centred on immunosenescence [22,51]. Here, we saw clear associations between *Selenomonas*, *Campylobacter*, and *Capnocytophaga* with T-cell immunosenescence, and together with the activated immature NK cell-associated *Alloprevotella*, these genera were all associated with periodontitis, gingivitis, and T2D. Diseases such as periodontitis an gingivitis have long been associated with changes in both the local and peripheral immune systems. This may be mediated by IL-17 from T_h17 cells, and it has been implicated in periodontitis-associated distal diseases in many disease contexts [90,91]. This is directly induced by microbial dysbiosis [92]. Furthermore, direct microbial interaction with immune cells may underlie this, as loss of Toll-like receptor-2 (TLR2) in antigen-presenting cells reduces IL-17 secretion from T_h17 cells that dysregulate the host immune system in periodontitis [93]. A similar direct link from the microbiome to the induction of a T_h17 cell response was previously reported for Streptococcus [94]. As such, it is interesting to hypothesise that innate immune signalling from TLRs on immune cells within the oral cavity may directly mediate microbiome–immune interactions, acting locally and distally.

As with all investigations, our study is not without limitations. Due to the limited quantity of the biobanked samples, 16S sequencing was favoured over shotgun sequencing to ensure good-quality data, leading to a limited taxonomic resolution in comparison to metagenomics studies. Future metagenomics studies are needed to refine the herein-presented association pattern, exploring potential differences within one genus. The EpiPath cohort consists of only 115 participants. This is a considerable number for a study on ELA, in which a full psychosocial stress test was performed, together with full immune and psychological profiling. However, this sample size is considered small for a microbiome studies, where statistical screening leads to multiple testing, reducing the statistical power for detecting individual associations. Similarly, the reported mediation analyses lack statistical power, and negative results should not be interpreted as the absence of effects. Similarly, as EpiPath is an adoption cohort, metadata such as the mode of birth, if they were ever collected, were never transferred to the adoptive parents. It is also possible that our data could be interpreted as the early inoculation with different microbiomes that simply persisted until 24 years later. The invasive nature of the ELA questions meant that compromise on microbiome-specific metadata, such as dietary habits and oral health status, was unavoidable if maximum participation in the study was to be ensured. Such information would have enhanced the mechanistic potential of our dataset. Knowledge of potential oral complications such as carries or periodontitis will be necessary in future studies to ensure that mechanistic pathways can be explored [95]. As the cohort consists of observational human data, causal interpretations of the reported associations should be treated with care. However, we demonstrated that 16S sequencing, despite its known limitations, provided clear insight into the long-term effect of ELA on the microbiome. Follow-up studies using shotgun metagenomics may refine the reported associations on the species and strain level.

4. Materials and Methods

Participants For this study, we used our previously reported EpiPath cohort of 115 adults aged 20 to 25 years [19,22,51,96]. A total of 75 control participants were brought up by their biological parents and 40 participants were adopted in Luxembourg from institutions worldwide. The median age at adoption was 4.3 months (IQR 0–15 months) [51]. Basic immunoprofiling was available for all cohort members [22,51]. Furthermore, detailed

NK cell profiling was available for 76 participants (19 cases and 57 controls), and immunosenescence profiles were available for 79 participants (19 ELA and 60 controls) [22,52]. Biobanked oral swabs were available for 98 participants (33 ELA and 65 controls) and buccal swabs for all 115 participants (40 ELA and 75 controls). For one participant without immunosenescence profiling, the body mass index and sex were missing. This individual was excluded from statistical analyses, where the BMI and/or sex were used as covariates.

Oral samples Saliva samples were collected using Salimetrics Oral Swabs (Salimetrics, Cambridge, UK). Salivary cortisol levels have previously been reported from these samples [19,96]. Buccal swabs were collected with Isoxelix Buccal Swabs (Isohelix, Harrietsham, U.K.). Microbial DNA was extracted using Qiagen DNA from a body fluids kit (Qiagen, Venlo, The Netherlands) according to the manufacturer's protocol. Samples were quantified with Qubit 1.2 (Invitrogen, Merelbeke, Belgium) and quality was assessed with a Nanodrop (Thermofisher, Merelbeke, Belgium). The V4 region of the 16S gene was amplified from bacterial DNA using 515F [97] and 806R [98] forward and reverse primers (Eurogentec, Seraing, Belgium). The amplification reagents and library preparation were performed using a Quick-16S kit and its equivalent dual indexes (BaseClear, Leiden, The Netherlands) using the manufacturer's low microbial DNA concentration protocol. Libraries were quantified with Qubit, 1.2, 1.4 (Thermofisher, Merelbeke, Belgium); quality and size were assessed using a BioAnalyser (Agilent, Diegem, Belgium). Sequencing was performed on an Illumina MiSeq system with v2 sequencing chemistry and 500 bp paired-end reads, as well as 10% PHIX control according to the manufacturer's protocol.

Bioinformatic analyses Fastq files were processed, aligned, and classified using mothur 1.41v [99]. Alpha (inverse Simpson diversity index and Shannon evenness index) and beta diversity (Jaccard Index) were further calculated in the same pipeline. Sequences classification was aligned based on the Silva v138 database [100]. Further integration of microbiome data into the immunophenotype and metadata as well as visualisations were performed with R.

Statistical analyses For descriptive statistics, nominal variables are described by proportions, while metric variables are described by means and standard deviations. Evenness and Shannon entropy metrics were calculated for the OM as measures of alpha diversity and compared between ELA cases and controls with Wilcoxon rank sum tests. Additionally, diversity measures were compared across the OM using rank correlations. For investigating statistical associations between taxonomical units and immune-cell numbers, relative abundances for all genera were checked for outliers. Observations that were outliers both in immune-cell numbers and relative abundances (more than four standard deviations away the mean) were excluded from the analyses, when analysing genus–immune-cell associations. Only genera, or phyla, detected in more than 50% of all cases, were analysed. The microbial abundance data were analysed using fractional regressions [101,102]. Fractional regressions are semiparametric methods not relying on distributional assumptions, and are specifically designed for the analyses of relative abundance data, making them suitable for the analysis of microbiome data, as different species abundances may not be sampled from the same class of distributions. Fractional regressions can be parametrised by odds ratios, allowing for easy interpretation of the regression coefficients in terms of the chance that a certain sequence read is assigned to a taxonomic unit [102]. All fractional regression models, if not specified otherwise, included age, BMI, and sex as covariates, and were performed separately for the OM communities. The basic covariates were included mainly to reduce residual variance and thereby increase statistical power to detect associations with the predictor of interests. Using fractional regressions, we screened the microbiome for associations with the study group variable, basic covariates (age, sex, body mass index (BMI), and smoking), antibody titres for Epstein–Barr virus (EBV), cytomegalovirus (CMV), and the herpes simplex virus (HSV), immunosenescence markers, as well as immune cell counts. All p-values are reported two-tailed. Statistical analyses were performed in STATA 16/MP (College Station, TX, USA), and correction for multiple testing was performed by applying the false discovery rate (FDR) [103]. An FDR < 0.05 was considered to be

significant. Summary statistics of the performed analyses are given in Supplementary Tables S1–S4.

5. Conclusions

Our data show a clear link between ELA and the OM that was visible 24 years later. The two oral communities investigated were clearly associated but distinct. We previously reported that ELA induced higher activation and senescence of the immune system. The taxonomic differences in the oral composition were not only associated with ELA but also with the immunosenescence of CD4 T cells, circulating numbers and activation status of NK cells, and anti-CMV titres. Although we do not yet have a detailed mechanistic explanation, our data suggest the presence of multiple links between ELA, immunosenescence, and cytotoxicity that persist through long-term changes in the microbiome.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/ijms222312682/s1.

Author Contributions: Conceptualisation, E.G.C. and C.P.M.; data curation, E.G.C. and J.H.; formal analysis, E.G.C., J.H. and J.D.T.; funding acquisition, C.P.M., I.T. and J.D.T.; investigation, E.G.C., S.B.M., P.G., F.A.D.L. and M.M.C.E.; methodology, E.G.C., S.B.M., P.G. and J.H.; project administration, J.D.T.; supervision, J.D.T.; visualisation, E.G.C.; writing—original draft, E.G.C., J.H. and J.D.T.; writing—review and editing, E.G.C., S.B.M., P.G., C.P.M., F.A.D.L., M.M.C.E., I.T. and J.D.T. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki revised in 2013, and approved by the National Research Ethics Committee of Luxembourg (Comité National d'Ethique de Recherche, CNER, reference 201303/10 v1.4). All participants provided written informed consent, and to compensate for time, effort, and inconvenience, all participants were reimbursed up to €150.

Informed Consent Statement: Written informed consent was obtained from all study participants.

Data Availability Statement: All data from the EpiPath study are available upon reasonable request to J.D.T.

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Conflicts of Interest: The authors declare that they have no conflict of interest.

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Appendix 2 – Contribution to co-authored publications

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Abstract: A poor socioeconomic environment and social adversity are fundamental determinants of human life span, well-being and health. Previous influenza pandemics showed that socioeconomic factors may determine both disease detection rates and overall outcomes, and preliminary data from the ongoing coronavirus disease (COVID-19) pandemic suggests that this is still true. Over the past years it has become clear that early-life adversity (ELA) plays a critical role biasing the immune system towards a pro-inflammatory and senescent phenotype many years later. Cytotoxic T-lymphocytes (CTL) appear to be particularly sensitive to the early life social environment. As we understand more about the immune response to SARS-CoV-2 it appears that a functional CTL (CD8+) response is required to clear the infection and COVID-19 severity is increased as the CD8+ response becomes somehow diminished or exhausted. This raises the hypothesis that the ELA-induced pro-inflammatory and senescent phenotype may play a role in determining the clinical course of COVID-19, and the convergence of ELA-induced senescence and COVID-19 induced exhaustion represents the worst-case scenario with the least effective T-cell response. If the correct data is collected, it may be possible to separate the early life elements that have made people particularly vulnerable to COVID-19 many years later. This will, naturally, then help us identify those that are most at risk from developing the severest forms of COVID-19. In order to do this, we need to recognize socioeconomic and early-life factors as genuine medically and clinically relevant data that urgently need to be collected. Finally, many biological samples have been collected in the ongoing studies. The mechanisms linking the early life environment with a defined later-life phenotype are starting to be elucidated, and perhaps hold the key to understanding inequalities and differences in the severity of COVID-19.

Keywords: COVID-19; SARS-CoV-2; socioeconomic status; early life adversity; psychosocial stress; immunosenescence; immune exhaustion; health inequalities

1. Introduction

The ongoing outbreak of coronavirus disease (COVID-19) was first reported in December 2019 in Wuhan, China. COVID-19 is caused by a betacoronavirus, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), that affects the respiratory system [1]. Despite draconian sanitary measures being applied worldwide, COVID-19 was declared a pandemic on 11 March 2020 by The World Health

of Organization (WHO) [2]. By May 13th the outbreak had infected over 4 million people and caused almost 300,000 deaths worldwide (World Health of Organization, 2020).

There is a long-established epidemiological observation that social adversity associates with reduced host resistance to infection and disease [3] which goes back as far as 1976 [4]. More recently, it was recognized that the effect on adult immune function and disease risk was much stronger when the exposure to adversity occurred during early life [5,6]. Humans are not fully developed at birth. Nervous and immune systems are gradually developed and educated up to the age of two. In fact, human life commences and develops for the first 1000 days starting from fetal conception. Any pre-natal complications and post-natal adversity faced defines the lifelong health trajectory [7]. As the COVID-19 pandemic has progressed, it has become clear there are many inequalities in susceptibility and severity of the disease. The recent flurry of pre-print clinical data from many countries worldwide including China, UK, US, are strongly concordant; the lower the current socioeconomic status (SES), the greater the risk [8], however, the role of the early life period and the resultant life-course has so far not been investigated. To understand the mechanisms underlying these differences, we need to dissect the exposome and environmental factors (i.e., pollutants, stress situation, etc.) that patients may be, or have previously been exposed to.

There is a well-established literature on the role of the overall trajectory from early life through to adulthood and the risk of non-communicable diseases such as cardiovascular disease, diabetes, obesity and depression [9], however there is no data on how it affects COVID-19. Although current SES has been associated with the risk, progression and even survival of non-communicable diseases [10], it is now becoming clear that during an individual's life there are periods of increased susceptibility, and the overall trajectory of SES may be more important. This has led to the "Barker theory", or the Developmental Origins of Health and Disease (DOHaD) [11]. In addition, environmental influences which act during early development/life may determine our susceptibility to the disease many years later [11–13].

Over time, the Barker theory has been refined. Currently, this is thought of as a "three hit model". The three "hits" are generally accepted as: (1) genetic predisposition, (2) early life environment and, (3) later life environment [14,15]. As high-quality mechanistic studies have addressed the link between the early-life period and adult disease, it is becoming clear that the immune system, particularly through chronic low-grade inflammation and accelerated immuno-senescence is, mechanistically, in the heat of the action. In addition, we know that stressful experiences during early life induce adaptive responses that are often mediated by the immune system [16].

In this manuscript, we examine the data linking early life adversity to life-long disturbances in the immune system that may play a role in determining its ability to fight SARS-CoV-2 infection, potentially determining the severity of COVID-19 disease and expanding DOHaD to cover infectious diseases later in life.

Furthermore, we review known factors of ELA and their potential influence on the adult immune system and contemplate what kind of data should be collected to understand how SES and ELA influence disease susceptibility and severity of COVID-19 and other diseases. We hope this work will contribute in protecting and treating people at risk of developing severe COVID-19 symptom.

2. The Role of Current SES in COVID-19 Morbidity and Mortality

Socioeconomic status (SES) or gradient is a combination of education, incomes, occupation and reveal inequities to privileges or resources between individuals [10]. Indeed, socioeconomic factors (i.e., race/ethnicity) are considered as fundamental determinants in human life span, well-being and health [10]. Data from influenza pandemics of 1918 and 2009 showed that socioeconomic factors may determine both disease detection rates and overall outcomes [17–19]. In the early phase of the COVID-19 pandemic many studies focused on basic criteria (i.e., age, sex, and gender) to investigate coronavirus spread, transmission routes and potential high-risk populations. Socioeconomic data were, unfortunately, missing as they are not considered as data of clinical interest [16]. However, socioeconomic data regroup many relevant factors as daily situations (i.e., stressful job, pollution, etc.)

that directly interact with human health [16]. Evidence is now starting to emerge that COVID-19 mortality is increased in ethnic minority populations. US data indicates that, for example, in Chicago approximately 70% of the deaths were from ethnic minorities [20]. Detailed data from New York showed that the number of COVID-19 cases associated with the percentage of dependents in the local population, the male:female ratio, and low-income neighborhoods [21]. United States-wide data gave a similar result, with proportion of residents >65 years old, ethnic minorities, male:females ratio, and the overall population density associating with increased frequency of COVID-19 [22]. The United Kingdom followed a similar profile. Although the recent UK data only looked at mortality, there was a stronger link between COVID-19 mortality and SES than ethnic background. A 1% increase in the lower socioeconomic class increased COVID-19 mortality by 2% (95% Confidence interval of 1% to 4%) while a 1% increase in ethnic minority increase mortality by only 1% (95% confidence interval 1% to 2%) [8]. Although these are preliminary (pre-print) data, they agree with Shi et al., who reported that the most severe cases were mostly agricultural laborers [23]. The link between the incidence of COVID-19 and lower income neighborhoods and lower SES is most likely due to the overall economic conditions such as poverty, performing essential public tasks, poor quality and over-populated housing as well as an obligation to use public transport [8] as well as higher rates of known comorbidities including type 1 and 2 diabetes, as well as cardiovascular disease and hypertension [24]. Overall, despite the scarcity of the data, we interpret what is available as a suggestion that current SES and neighborhood influence the morbidity of SARS-CoV-2 infection and COVID disease rather than the mortality rate.

3. The Role of Early Life in Determining Lifelong Health Trajectories

When considering the early-life environment, many measures such as SES are broad and encompass many concurrent elements. We have previously found it useful to separate these into four principal sub-categories [15] (Figure 1). Although determining the contribution of each of the four elements (psychosocial stress, infection, nutrition and microbiome, and pollutant exposure) is difficult, there are data on well-defined exposure conditions that fit into these sub-categories as well as insidious, general measures like SES.

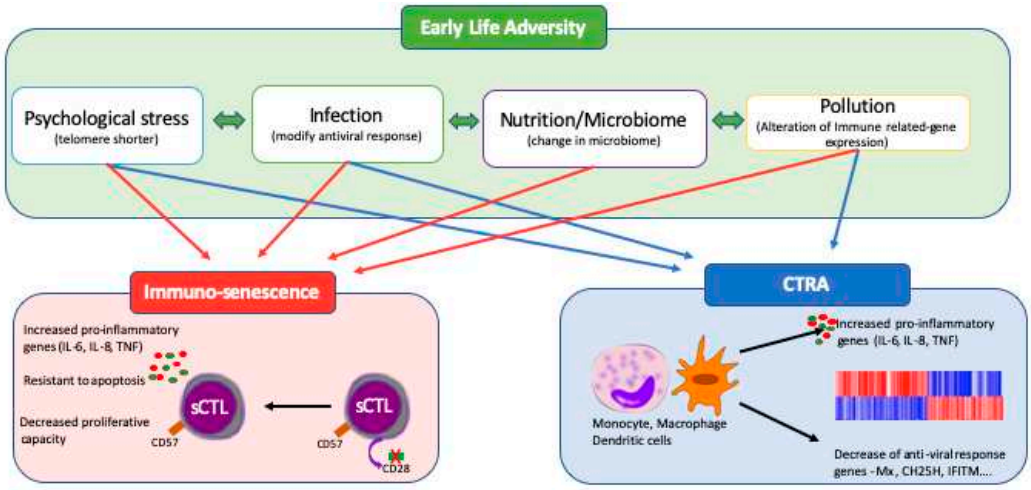

Figure 1. Immune adaptation mediated by early life adversity. Early-life adversity (ELA) is broken down into its four key components: psychosocial stress, infectious stress, nutrition and the microbiome; and pollutant exposure. They are linked to increases in the numbers of senescent cytotoxic lymphocyte (sCTL) which, upon stimulation are resistant to apoptosis and release large quantities of expression of pro-inflammatory. Certain elements have also been shown to alter the underlying transcriptional identity of leucocytes such as macrophages, dendritic cells or T lymphocytes. This phenomenon is called "the conserved transcriptional response to adversity" (CTRA).

3.1. Early Life Psychosocial Stress

There is now a growing literature on the effects of early-life psychosocial adversity on the immune system. We have previously reported the immunophenotype of young adults that had experienced ELA as institutionalization after separation from their parents and subsequently adopted in early childhood compared to those reared by their biological parents (EpiPath cohort) [25]. In this cohort, we surveyed the innate, humoral, and adaptive immune system. We observed an increase in activated and senescent pro-inflammatory T cells, particularly those, expressing HLA-DR/CD25 and CD5. Senescence is a natural aging process affecting all cells including immune cells. These begin to deteriorate and this leads to weakened immune responses [26]. Furthermore, there was a trend toward an increase in the number of circulating Th17 cells [27,28]. ELA clearly accelerated T-lymphocyte maturation and senescence, although did not affect B cells. T- lymphocytes were accelerated through their maturation cycle from naïve to effector memory and aggregating in the terminally differentiated effector memory cells re-expressing CD45RA (TEMRA) cell phase [27,28]. This skewing of the immune system, in particular the cytotoxic CD8+ T-cells was confirmed in an independent cohort, of teenagers approximately 15 years after a similar form of ELA [29].

Telomere length decreases with chronological and biological age, after cell division, and is a hallmark of cellular senescence. Exposure to stressful events during childhood showed that the telomere length is shorter in these individuals when compared to the control group [30–33], confirming that ELA negatively contributes to an imbalanced immune system [34]. Furthermore, Cohen et al. showed that low childhood SES significantly decreased the telomere length later in life of a CD8+CD28- T cell population, which play major role in the response to viral infections [35].

Studies with rodents produced the predominant hypothesis that the mechanism by which ELA impacts the function of CD8+ cells and, consequently, viral responses, may be through the HPA axis. ELA negatively impacts the HPA axis, which programs its effects and responses later in life. This normally results in a decreased release of corticosterone or cortisol after exposure to stress which consequently has a great impact on the peripheral immune system, leading to compromised viral responses [36–39]. However, results from mechanistic studies in our EpiPath cohort have excluded this. We were able to show that despite an altered HPA axis [25], glucocorticoid signaling and the peripheral HPA-axis stress system were not epigenetically programed [40], implying that the immune system was directly impacted.

3.2. Early Life-Infections

It is well known that an early life exposure to infection and inflammation can have devastating effects. One example would be that neonates suffering from bacterial or viral sepsis are about threefold more likely to die within the first 120 days [41]. There is also evidence showing that sepsis in new-borns was associated with poor long-term neurodevelopment [42]. The immediate risk of infection to the organism, especially for those more vulnerable, seems obvious. The long-term consequences of an infection prove far more difficult to grasp.

Bilbo and Schwarz reviewed available data on the connection between perinatal infection and long-term effects on stress reactivity and cytokine production [43] showing that early life infection leads to a cytokine storm (the most prominent being interleukin 1β [IL-1β], IL-6 and tumor necrosis factor α [TNFα] which can pass the blood-brain-barrier and cause long term memory impairment in the hippocampus. Similarly, we found a blunted response to stress and a higher number of exhausted T-lymphocytes in our EpiPath adoptee cohort, which had a higher incidence of cytomegalovirus (CMV) infection and an overall higher risk of childhood infections due to the institutionalization [25,27]. A very recent study in zebrafish shows that expression of several pro-inflammatory genes is increased in adult fish after early life bacterial infection [44]. This study also showed that the age of the first infection is a crucial factor for the adult immune response. Other studies have specifically linked early-life respiratory viral infection with a higher likelihood to develop diseases like childhood asthma

or allergies [45–47] or the chance to develop type 1 diabetes [48]. These chronic conditions are known risk factors for a more severe outcome of COVID-19 disease.

Currently, the molecular mechanisms in which an early life infection distorts the immune system are only partially understood. In in-vitro experiments, Fonseca et al. demonstrated that early-life exposure to bacteria in combination with respiratory syncytial virus (RSV) later in life can lead to epigenetic modifications impacting bone marrow progenitor cells and therefore causing long-term re-shaping of inflammatory mediators and metabolic profiles [49]. Subsequently, all daughter cells of these progenitors would be ill-equipped to handle subsequent infections [47].

Certainly, early life infections present a specific type of early life adversity. It is indubitably linked to the overall health of the individual (immune system) and the social environment, given that host-to-host transmission of pathogens are by far the most prevalent form of infection. In the previous section, we showed the impact of psychosocial stress on the immune system. However, the overlap does not end there: sickness, in humans and animals, also changes their social behavior. Well known behavioral changes include a decrease in activity and expanded sleeping periods [50]. Therefore, social behavior and infection should not be treated as two distinct adversities, but as two sides of the same coin.

Early life nutrition and the microbiome: Over the last decade it has become clear that once the microbiome is established it is shaped by the exposome and the ~9 million microbial genes it encodes and play a crucial role in determining host development and health [14,51–53]. Modulating the host most probably protects the natural enteric symbiotic microbial community, and disturbing the established microbiome, producing a dysbiosis, results in disease and may even be fatal [54,55]. The microbiome established is dependent on the route of birth, and is then modulated by nutritional intake, living conditions, the polluted environment and the presence of pets [56,57]. As SARS-CoV-2 appears to persist in the GI tracts and can be detected in human feces [58,59], it will interact, affect, and be affected by the microbiome. Indeed, diarrhea is now recognized by the Centers for Disease Control and Prevention (CDC) as a COVID-19 symptom and it is a clear sign of microbial dysbiosis [60]. The interaction and effects of SARS-CoV-2 will almost certainly depend on both the microbiome that has been established and how the host has adapted to its microbiome.

The LPS content and immunostimulatory potential of the initial early-life microbiome depends on the birth route [51]. The microbiome is established during a sensitive period in which the new-born immune system is primed [61], and may explain why babies born by caesarean section have a significantly increased risk of allergy or asthma later in life [62]. Exposure of new-borns to a more diverse microbiota soon after birth altered both the disease susceptibility and maturation of specific immune cell subsets, whereas if the first encounter occurred later, immune dysfunction was not corrected [63,64]. Regulatory T cells (T_{reg}) play a significant role in the host adaptation to the microbiome, recognize host-specific commensal bacteria derived antigens [65], and result in long-term tolerance to the enteric microbiome [66]. It would appear that adverse microbiota is essential for the immune system to fully mature [67].

Peri-natal viral infections, such as CMV have been extensively studied and linked to lifelong changes in the microbiome [68] and common viruses such as influenza are known to affect the development of the immune system when acquired at birth and during infancy [69]. The angiotensin-converting enzyme 2 (ACE2) receptor may play a role in determining microbiome-immune-interactions. In the GI tract ACE2 is expressed in enterocytes and is important for maintaining both antimicrobial peptide expression, and the overall health of the microbiome [70,71]. Mice lacking Ace2 develop gut absorption related diseases [70,72]. As Sars-Cov-2 uses ACE2 receptor to enter cells [73,74] it would be logical to assume that there is a link between the virus and the microbiome that was established in early life, immune cells resident in the GI tract and the overall outcome of COVID-19.

Early life-pollution exposure: There is emerging evidence that environmental exposure to pollutants during sensitive developmental periods like early life could be a strong factor of susceptibility,

predisposing the individual to birth outcomes and disease onset in later life [15]. Prenatal exposure to airborne pollutants could affect fetal reprograming by epigenetic modifications (e.g., DNA methylation) and may therefore explain the potential link between air pollutant exposure and adverse pregnancy outcomes. Epidemiological studies have pointed out causal association between fine particulate matter (2.5 μm; PM2.5) and neurodevelopmental (ADHD, autism)/neurodegenerative (Parkinsons, Alzheimers) [15], metabolic, cardiovascular [75] and lung pathologies [76]. Air pollutants were therefore proved to affect key cellular/molecular targets during the perinatal period, which are susceptible to alter immune responses link to abnormal respiratory functions and lung diseases later in life [77]. For instance the EDEN birth cohort study, focusing on determining peri-natal factors that influence childhood health and social development, pointed out that a pre-natal exposure to PM10 (particles with diameter less than 10 μm) was linked to an increased in CD8+ T cell and a decreased in regulatory T cells in infants at birth, leading to a potential increase in the susceptibility of viral infection responses as well as atopy development in children [78]. The impact of traffic pollutants and tobacco smoke on regulation of numerous Immune related-genes, such as cytokines (e.g., IL-4, IL-6, and IFNg), TLR2, nitric oxide synthases (NOSs), and several factors of transcription (e.g., Runx3 and Foxp3), has also been demonstrated [77]. It is now well established that modifications in DNA methylation patterns due to PM 2.5 exposure are frequently associated with the development of lung pathologies [79]. However, it remains difficult to assess whether exposure during early life has a stronger impact on development of diseases than that of the adulthood, or whether substantial morbidity is the result of accumulated exposure [76].

In the context of COVID-19, Zhu et al. demonstrated significant associations between air pollution and COVID-19 infection. High concentration levels of $PM_{2.5}$, PM_{10}, CO, NO_2 and O_3 were therefore positively linked to a risk of COVID-19 infection, whereas high concentration levels of SO_2 were negatively linked to the number of daily COVID-19 confirmed cases [80]. These results are supported by those obtained in February 2020 by Martelletti et al., who showed that in the industrialized regions of Northern Italy, those most affected by COVID-19, the concentration levels of PM10 and PM2.5 were above the legislative standard limit of 50 μg per day [81]. The adsorption of SARS-CoV-2 RNA on airborne PM (PM2.5 and PM10) was established in these regions by Setti et al. who suggested that, "in conditions of atmospheric stability and high concentration of PM, SARS-CoV-2 could create clusters with outdoor PM, and, by reducing their diffusion coefficient, enhance the persistence of the virus in the atmosphere." [82]. In a cross-sectional observational study conducted in the United States, Wu et al. showed, by taking into account 20 potential confounding factors in their main analysis, that a slight increase in PM2.5 (+1 $\mu g/m^3$) was linked to an 8% increase in the rate of COVID-19 death [83]. Although all this data results from preliminary investigations, it tends to suggest a positive relationship between ambient air pollution exposure and COVID-19 mortality rate. Confirming the direct impact of airborne pollutants on the COVID-19 severity could prove an asset in terms of public health and prevention strategy in places with poor air quality.

We have previously highlighted the role of early-life pollution exposure and a potential "second hit" in the "three-hit" model producing a quiescent phenotype, likely encoded in the epigenome, which might become vulnerable in later life to a "third environmental hit" such as COVID-19 [15]. Given the long-term effects on health of early-life pollutant exposure and the linkage with the development and progression of pulmonary pathologies in later-life, it is reasonable to assume that early-life pollutant exposure will affects the course of COVID-19.

4. Early Life Origins of COVID Co-Morbidities

If the early life environment plays a role in determining the outcome of COVID-19, examining its role in the key comorbidities is essential. The three key comorbidities determining COVID-19 severity are cardiovascular disease, hypertension and diabetes. The seminal work of David Barker clearly identified the role of the in-utero environment, another source of early life adversity, in determining the risk of both cardiovascular disease and hypertension. While this has been extensively reviewed elsewhere [84–86] it is worth noting that the relative risk associated with birthweight and ponderal index is by far larger than

any other risk factor identified for either disease to date. There is now a large body of evidence showing diabetes to be a major risk of complications and death after SARS-CoV-2 infection [87], as in previous coronavirus outbreaks [88], while the risk of SARS-CoV-2 infection appears to be similar [89]. Like the other elements discussed here, type 2 diabetes (T2D) may have its origins in early life. There are well-established, classical risk factors that contribute to T2D including obesity, age, stress, inflammation, diet, lifestyle and environment (both early and late life), however there is growing recognition for non-classical factors such as pollution, exposure to ionizing radiations and low socio-economic status (SES). The classical and non-classical factors are intimately intertwined. SES is a broad measure encompassing prior life history, and low SES also increases the risk for obesity, stress, environmental and lifestyle factors (BMI, smoking, alcohol . . .) as well as a pro-inflammatory phenotype [90].

The importance of T2D in determining COVID-19 severity may in part be due to treatment strategies currently used in T2D together with another severe co-morbidity, hypertension. Both are often treated with ACE (angiotensin converting enzyme) inhibitors and ARBs (angiotensin II receptor blockers). These increases ACE2 (angiotensin converting enzyme 2) expression in pancreatic islets, lungs, intestines, etc. [91]. SARS-CoV-2 exploits these ACE2 receptors to enter host cells, thus potentially increasing the risk of infection in T2D patients [92]. Increased pancreatic ACE2 activation has been reported to inflict beta cell damage complicating the prognosis [93] and further contributing to the characteristic "cytokine storm" observed in COVID-19 cases. Other T2D drugs that induce ACE2 expression include pioglitazone, liraglutide, gliflozins, and DPP4 (dipeptidyl peptidase 4) inhibitors and have also been implied to promote coronavirus predisposition [94]. This may be further accentuated by hyperglycemia-induced ACE2 glycosylation. ACE2 glycosylation is also a prerequisite for the virus to latch onto the ACE2 receptors [95]. This enhancement is reversible by strict glycemic control [95]. As such, glycemic and overall diabetic status have been proposed as predictors of COVID-19 severity and mortality [96].

Although current T2D status may play an important role in SARS-CoV-2 susceptibility and COVID-19 severity, it is part of a larger etiopathological risk complex. T2D may have its origins in the early life social environment. Low early-life SES showed a clear, strong, association with individual metabolic profiles that was not true for current SES [97]. This result has been replicated by another study that highlighted the effect of SES during adolescence on the development of T2D up to fifty years later [98]. More recently, Chandan et al. (2020) reported a retrospective population-based cohort of 80,657 adults that had been exposed to ELA and 161,314 unexposed controls. This seminal study clearly demonstrated the link between childhood maltreatment and cardiovascular disease, hypertension, and T2D. In a population where ELA rates may reach 25%, their data clearly shows that "a significant proportion of the cardiometabolic and diabetic disease burden may be attributable to maltreatment" [99].

There is now some mechanistic evidence to back up the link between ELA and T2D. Needham et al. investigated the transcriptional effects of low SES [100]. They reported that low (current) adult SES altered the expression of several genes intimately linked to inflammation that are all linked to T2D: *F8* [101], *CD1D* [102], *KLRG1* [103], *NLRP12* [104], and *TLR3* [105] and stress related gene *AVP* [106]. Furthermore, low early-life SES was also shown to affect the expression of stress related genes: *FKBP5* [107] *OXTR* [108] and *AVP* and inflammation associated genes: *CD1D* and *CCL1.* As such, SES would appear to act on inflammatory pathways that are common to low SES environments and eventually T2D, and may worsen the T2D etiopathology by targeting prominent pathophysiological factors like stress and inflammation. The mechanistic link between ELA and T2D is re-enforced by the immune disturbances reported. Patients with T2D have a larger number senescent CD8+ cytotoxic T cells and higher levels of systemic inflammation [109,110] that may explain the higher incidence of viral and bacterial infections in diabetic patients [111].

Although there is no data currently available, it is logical to assume that although T2D may predict COVID-19 severity, the origins of this link may lie in the lifelong pro-inflammatory environment

induced by ELA. T2D may be the adult manifestation of the poor early life social environment which then mediates the effect between ELA and COVID-19.

5. The COVID-19 Immune Response, SES and Early Life Adversity

The immune response to COVID-19: The SARS-CoV-2, like other viruses, is considered immunologically as an intracellular parasite. In general, the viral infectious-cycle starts with a short-lived extracellular period, followed by cell entry, with a final, longer, intracellular replicative period. In the classical anti-viral immune response, the immune system attacks all phases of the viral cycle using both antigen specific and non-specific mechanisms. The non-specific immune response, particularly effective in the early phase of infection, is mainly mediated through natural killer cells and interferons. Production and/or secretion of type-1 interferons (i.e., all the interferons proteins except IFN-γ) enhances NK cell ability to lyse infected cells as well as inhibits viral reproduction and cellular proliferation. When an adaptive immune response has been mounted, the most effective antibodies are the so-called neutralizing antibodies which block viral entry into the host cell by binding to viral surface proteins such as the envelope or capsid protein (Figure 2). When the subsequent cell-mediated immunity enters into force, it is principally CD8+ cytotoxic T lymphocytes (CTLs) that are the effector cells. CTLs recognize MHC class-I presented antigens, to lyse the presenting cell, a response that is not always beneficial as the damage done by the cytotoxic cells is occasionally greater than that of the virus itself.

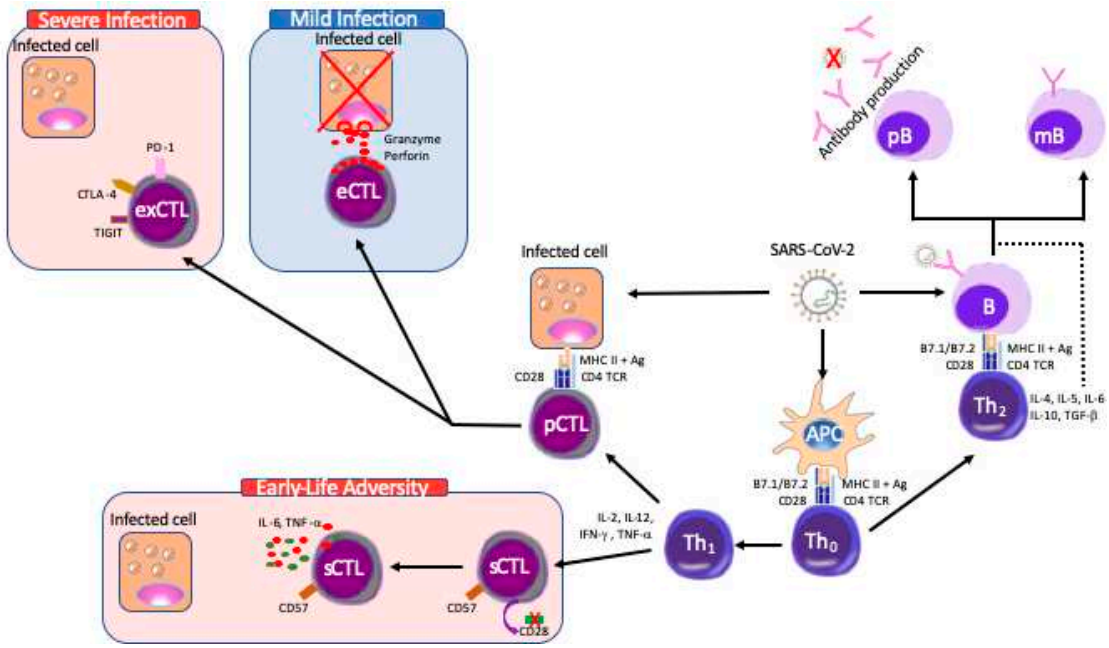

Figure 2. The immune reaction to coronavirus disease (COVID-19). The adaptive response to SARS-CoV-2 is a classical anti-viral response. On the right side, once recognized by antigen presenting cell (APC), Th_2 response is activated and induced maturation of B cell. After maturation precursor B cell produces a specific antibody against SARS-cov-2 while mature B cell retain memory of SAR-COV-2 to produce antibodies in case of new infection. Once the Th1 system is activated it induces activation of precursor cytotoxic lymphocyte T (pCTL) due to expression of many cytokines (IL-12, IL2). In one hand, effector (eCTL) can release proteins as granzyme to destroy infected cell in case of mild infection. In case of severe infection, CTL become exhausted (exCTL) and express PD-1, TIGIT and CTLA-4. In patients with having experienced ELA, the increased relative number of sCTL having lost CD28 expression will produce a less efficient lysis of SARS-CoV-2 infected cells. The recognition and clearance by NK cells and the initial role if Interferons is omitted for clarity. Cell images were from http://www.clker.com with the right to re-use them.

As the COVID-19 pandemic has progressed, there have been several reports of the anti-SARS-Cov-2 immune response. To date, the data suggests that the response is a classical anti-viral response with activation of Type-1 interferons and CD8+ CTLs. Although Thevarajan et al., analyzed a single patient, they nicely demonstrated the kinetics of the anti-SARS-CoV-2 immune response [112]. In a manner similar to both Influenza infection and a previous SARS-CoV-2 report [113] which showed that the numbers of $CD38^{+}HLA\text{-}DR^{+}$ $CD8^{+}$ T cells were higher in infected patients than in healthy controls, and rapidly increased from 3.57% (day 7), 5.32% (day 8) to a peak at 11.8% 9-days later. By day 20 they had decreased slightly to 7.05%. As would be expected, $CD38^{+}HLA\text{-}DR^{+}CD8^{+}$ CTLs, produced significant quantities of the lytic moieties—perforin, granzyme A and granzyme B—necessary to lyse virus-infected cells (Figure 2). Their kinetic data showed that this occurred at days 7–9, preceding symptom resolution, suggesting an important role in the resolution of the SARS-CoV-2 immune response [112].

The anti SARS-CoV-2 immune response in severe/critical patients: COVID-19 patients are generally considered either mild, severe, or critical. There are now data on the differences in the immune response in these different categories, although the categories are not always the same, complicating comparisons between studies. When Zheng et al. investigated T-cell derived functional molecules, they highlighted lower levels of interferon-γ (IFN-γ) and TNF-α in CD4+ T cells in severely affected patients than those mildly affected, although in the latter, they were considerably higher than expected in health controls [114]. Levels of perforin and granzyme B cells were increased in CD8+TIGIT- CTLs, and the numbers of senescent HLA-DR+ TIGIT+ CD8+ cells were increased in severely affected patients than those with a mild infection. The authors proposed that their data suggests COVID-19, like many chronic viral infections, reduces CD4-Tcell functionality, skewing the immune response towards a CD8+ response, with excessive activation leading to exhaustion of the CD8+ cells, diminishing the anti-viral immune reaction. Furthermore, upon deeper examination, they found differences in PD-1, CTLA-4, and TIGIT–markers of immune exhaustion. In severely affected patients, exhausted PD1+CTLA-4+TIGIT+ cells were significantly more frequent than in patients with a milder infection. This excessive CTL exhaustion may reduce the effectiveness of the immune response to SARS-CoV-2, explaining case severity [114]. Furthermore, in an independent study, it was also reported that as disease severity increases, the numbers of naïve, effector and memory classes of CD8+ T cells diminish, while B-cell, and CD4+ T cell numbers generally increase [115,116]. Overall, we interpret these data as showing that a functional CD8 response is required to clear SARS-CoV-2 infection, and COVID-19 severity is increased as the CD8+ response becomes somehow diminished (Figure 2). Indeed, Omarjee et al. have also come to a similar conclusion, that "Severe COVID-19 can therefore mimic a state of immune senescence" [117,118]. From the start of the pandemic, the involvement of the cytokine system was clear [119]. Initially described in January 2020, levels of CXCL8 and IFNγ, were increased in all COVID-19 patients, and severe cases had significantly higher levels CXCL10, CCL2 and TNFα than milder cases [120] reproduced in a more recent study that also observed increased levels of IL6, and IL 10 in the most severe cases [121].

Does Immunosenescence link ELA to COVID-19 outcomes? We have outlined above the ELA-induced long term immunophenotype. Although the origins are multifactorial, it would appear, from the work of Elwenspoek [27,28] and Reid [29], that an adverse social environment in early life drive T-cells, in particular CD8+ CTLs, towards a senescent state. When the different aspects of ELA are considered separately, immunosenescence would appear to be a common aspect. Senescence and exhaustion may have similar outcomes, a reduced immune reaction, but are distinct processes [122]. Senescent cells have a significantly reduced capacity to proliferate, however, they have a strong pro-inflammatory action. In a manner reminiscent of the senescence associated secretory phenotype (SASP) initially established in fibroblasts [123] senescent CD8+ CTLs aggregate in the highly differentiated states (effector memory and TEMRA), are highly resistant to apoptosis, and produce significant quantities of pro-inflammatory cytokines such as IL6 and TNFα upon stimulation [124]. Exhausted CD8+ CTLs

however, are not only unable to proliferate, but they no longer secrete cytokines after stimulation and are programed to undergo apoptosis.

The data currently available suggests that the aggregation of senescent CTLs will negatively impact the progression of COVID-19, and patients with the most senescent CTLs will have the poorest prognosis as they are less capable of mounting an effective CD8+ response, and they will have an exaggerated cytokine secretion from the senescent cells. This is further supported by the recent initiation of the SCOPE trial, "Sirolimus Treatment in Hospitalized Patients With COVID-19 Pneumonia" (NCT04341675). In this trial, the investigators propose administering rapamycin to down-regulate the IL-6 pathway through the mTOR pathway to not only reduce IL-β levels, but reduce the number of senescent T-cells as well [117]. This also raises the question about what happens to COVID-19 when ELA-induced senescence and COVID-induced exhaustion converge. It would seem logical to hypothesize that this would represent the worst-case scenario, and would produce the least-effective cytotoxic T cell response.

The Conserved Transcriptional Response to Adversity (CTRA): Studies have demonstrated that early life social adversity can act mechanistically through modifications of gene expression patterns. Gene expression implicated in the activation of T-lymphocyte and inflammation was enhanced while gene expression implicated in innate antiviral responses induced by type I IFN and innate antimicrobial responses of pathogen-specific was reduced [125]. These patterns of altered gene expression remain lifelong [125]. The pattern has been termed the conserved transcriptional response to adversity (CTRA), and has been noticed in many correlational studies regarding humans encountering with adverse life circumstances. [126–133]. CTRA dynamics are most strongly induced by social conditions in early life, at the first step of the development of postnatal immune system [125]. To the extent that transcriptome remodeling induced environmentally continue to affect immune responses of implicated pathogen, many, many years later in life (e.g., inhibiting immune responses to viral infections [134], or amplifying allergic inflammation [133,135]).

Essential co-variates: ELA is, however, associated with a range of negative health behaviors (reviewed in [136]) including an increased risk of smoking as well as increased smoking levels, levels of alcohol consumption, and poor diet leading to either malnourishment or obesity. The psychobiological and neurodevelopmental mechanisms linking ELA and risky health behaviors are starting to be dissected [137]. However, in the context of the COVID-19 pandemic, it would appear from the numerous studies that are becoming available that smoking increases the risk not only of hospitalization with COVID-19, but with ICU admission and death (odds ratio from 2.0 to 16 [138,139]) and was confirmed in recent meta analyses of the available studies [140–143]. On the other hand, there is little evidence available on the role of prior alcohol intake on the course of SARS-CoV-2 infection, however, considerable public health efforts are being made to combat alcohol abuse during the confinement period, and a prior history of ELA exposure may increase the risk of excessive alcohol consumption during this period.

Biological sex is one of the strongest drivers of the heterogeneity in COVID-19 disease severity. There is a clearly more favorable outcome for women across all age categories. The data available so far suggests that sexual dimorphism in the immune system may play a role in determining disease outcome. Sex impacts not only the development of T_{reg} cells, but the distribution of lymphocyte subsets and the overall T-lymphocyte response to challenge [144]. Many immunologically important genes are found on the X-chromosome including CD40L and CXCR3. Incomplete X-inactivation or epigenetic modifications will induce sex-specific effects on T-cells [145,146]. There is also evidence that there is a stronger lymphopenia in males than females in severe COVID-19 disease [147,148]

There is also growing evidence for the role of vitamins D and K in the outcome of COVID -19 disease. Beyond its classical role in bone metabolism [149], vitamin D plays a role in the functioning of the immune system and in the regulation of inflammatory cytokines [150] and CRP [149] which reduces the risk of infection and cardiovascular disease [149]. Indeed, immune cells like T-cells, B-cells or antigen presenting cells can directly interact with vitamin D receptors. In this way, increased vitamin

D levels enhance the innate system and suppress the adaptive immune system, which demonstrates its role in immune regulation [151]. Vitamin D deficiency is also linked to comorbidities such as diabetes [152] and upper respiratory disease susceptibility, including common viral infections, allergies and airway inflammatory conditions (REF6). The logical assumption is that a possible explanation on the susceptibility of the elderly population is the fact that they naturally produce less vitamin D while they are exposed to less sunlight as many stay indoors. Considering also that the pandemic first made its global appearance during winter season increases the possibility for this correlative association [152]. Panfili et al. highlighted the potential that vitamin D supplementation has shown to be a successful cost-effective therapeutic for acute respiratory tract infections (ARTIs) in low socio-economic characterized countries [153]. In addition, studies have shown that vitamin D can help to reduce the risk of an activated renin-angiotensin system in the lung [154] in cases of severe COVID-19 disease in patients with hypertension and high expression of ACE2 receptors [155]. On the other hand, patients with comorbidities such as diabetes present a lack in vitamin K which is involved in blood coagulation or bone calcification mechanisms. In case of COVID-19 patients, insufficient levels of vitamin K could be associated with a risk of complications due to elastic fiber pathologies such as idiopathic pulmonary fibrosis (IPF) [156]. Coagulation has been reported as a common comorbidity linked to COVID-19 severity and mortality.

6. COVID-19 as a Natural Experiment

Given the obvious ethical objections to experimental studies manipulating the early life environment, there is a long history of using natural experiments. There are two classical natural experiments looking at the early life social environment, Project Ice Storm in Canada, and the Dutch Hunger Winter. When we look at these natural experiments in the light of the three-hit model, these examined the role of the second hit, the early life environment.

Project Ice storm is based on the 1998 Quebec ice storm and examines the impact of prenatal stress on adult outcomes. This particularly harsh meteorological event affected, residents of a well delineated area covering Nova Scotia, New Brunswick, Southern Quebec and eastern Ontario. These populations had to deal with a situation where they were deprived of electricity for weeks, and in certain cases months, as well as the shutdown of all activities in major cities (Montreal, Ottawa) as well as military deployment and several deaths. Project Ice Storm went on to examine the effects over the following 20 years on the children and now young adults that were exposed to the storm in utero [157,158]. They concluded that prenatal glucocorticoid exposure impacted a variety of outcomes in the next generation throughout childhood and persisting into adulthood, dysregulating metabolic pathways and the HPA axis [157,159] This was mediated through epigenetic (DNA methylation) encoding of the storm's effect [158]. Project Ice Storm demonstrated that an environmental stressor can have long-term effects and inducing numerous outcomes although there were additional mechanisms linked to socioeconomic factors that are still to be identified.

The Dutch Hunger Winter was the consequence of a food embargo placed on the Dutch population by the Germans at the end of world war II [160]. Here, the importance of timing of the adversity in the programing of adult disease was established [161]. Working on same-sex sibling pairs of which only one was exposed to famine they demonstrated that in utero exposure induced an adverse metabolic [162] or mental phenotype [163], depending on the time of exposure and fetal sex, and that this was mediated by DNA methylation [164].

As Project Ice Storm disaster and the Dutch hunger winter, the current COVID-19 pandemic must be considered as a relevant natural experiment to reveal the effects of socioeconomic factors on health and disease. In the context of the three-hit model, here we have an exquisite and unique opportunity to investigate the third hit. As outlined above, the early life period acts through underlying mechanisms such as DNA methylation and programing of the immune system to influence disease progression and severity later in life. These prior studies have provided unexpected mechanistic insight into the immunological consequences of early life stress exposure. Drawing parallels with COVID, if we

can collect the correct data, we can start to unpick the role of the whole life trajectory and how this contributes to disease risk through a pro-inflammatory immune bias.

COVID-19 may also be a form of early life adversity. It is yet to be discovered whether SARS-Cov-2 could have any immune programing capacity after an early life infection and what consequences could appear years later. Its strong association and impact on the early life microbiome is unknown. Pregnant women who tested positive for SARS-CoV-2 infection showed evidence of placental injury which impeded blood flow to the fetus [165]. Placental development is the first step in embryogenesis and may determine the quality of the intra-uterine environment [165,166]. Individuals who were exposed (intra-uterine) to the Spanish flu of 1918 have been reported to face lifelong low SES and cardiovascular diseases [167] which may be indicative of a bidirectional risk that has crossed over from the placenta jeopardizing their lifelong health profile. It is quite possible that the COVID-19 positive mothers pass on a similar risk to subsequent generations, serving as an ELA event, which ultimately makes them highly susceptible. Thus, these cases need strict follow up studies to validate this hypothesis.

7. Data that Should Be Collected

In light of the data presented here, it is clear that there are many types of data that should be collected in addition to the studies that are currently ongoing addressing the epidemiology and biology of COVID-19. As recently highlighted, it is essential to collect as much socioeconomic data as possible during the ongoing pandemic [16]. Data collection should be expanded to include retrospective data on life-trajectories and both exposure to adverse life events and how importantly they were perceived. There are well-recognized difficulties in retrospectively assessing adversity or the overall life-course, however, there are tools available that can measure the prior traumatic experiences. Recent adult trauma can be addressed by a brief questionnaire that covers the perceived importance (salience) of a range of stressful life events including "separation, relationship and money worries, accidents, illness and death, job loss, and violence" [168] that any future study participants may have experienced. To address traumatic experiences earlier in life, there are also validated questionnaires such as the Childhood Trauma Questionnaire CTQ or the Early Trauma Index that are available [169]. However, as with any retrospective study there is a risk of recall bias, although the validated questionnaires have questions within them to ensure internal consistency. Furthermore, in the context of a fast-moving pandemic, the ability to transpose such questionnaires to an online system is known to improve the accuracy of responses as the anonymity of the online process has been shown to reduce both social desirability and central coherence biases, although there is a potential risk of questions being mis-interpreted by participants [170]. All such tools are limited by what was thought of as being traumatic when they were developed, however, they remain the standard tool for assessing traumatic events during childhood as well as a poor social and familial environment [169]. The use of such questionnaires has already proven useful. Adverse social conditions, as measured by the CTQ have been shown to become embedded as functional changes in the immune system that are visible lifelong. Studies have shown adversity measured by the CTQ over a period of as little as 4 months changes the immune response up to 24 years later, the longest time-point investigated so far [27,28,34,125]. Tools such as the CTQ should play a role in studies addressing the overall disease severity if participants go on to develop COVID-19 rather than whether ELA plays a role in the overall prevalence of infection. Furthermore, health related behaviors such as smoking and alcohol consumption which are known to be elevated after ELA and may also play a role in the clinical evolution or susceptibility to SARS-CoV-2 infection must be recorded. All data should be analyzed with a sex-informed approach, taking differences in the immune system into account.

The collection of life-event meta-data must be complemented by the collection of the correct biological samples. We have highlighted the role of the immune system, the microbiome and pollution levels. It would seem logical to obtain stool and blood samples, and the markers to be investigated such as TIGIT, PD-1, CD28 and CD57 are now becoming clear. Furthermore, such biosampling would allow the analysis of vitamin levels, as they may be a key link in the pathophysiological chain. It would

also appear to be appropriate to rapidly collect measures of pollutants, determine how indoor and outdoor pollution levels have changes, how, with the strict confinement measures imposed, nutrition has changes. All of these will play into the susceptibility and immune response.

The data reviewed here highlights the role that the social environment will play in determining morbidity and mortality during the COVID-19 pandemic. In the future, such socioeconomic and lifestyle data must be considered as essential clinical data that is then analyzed concurrently with biological material to tease out the effects of the environment in health and disease.

8. Conclusions

The developmental origins of health and disease is firmly established for many non-communicable diseases. The current COVID-19 pandemic has shown that there are many health disparities, and the available (preliminary) data suggests that there is a strong socioeconomic impact on morbidity, and potentially mortality. Although there are no data so-far available to link the early life period to the morbidity and mortality of an infectious disease, an adverse early life environment would appear to impact the immune system and make it less efficient in fighting subsequent viral infections. Early-life researchers have a long history of taking advantage of natural experiments, teasing out the long-term consequences of ELA to produce a measurable phenotype many years, or even generations, later. The current pandemic can turn this paradigm on its head. Many discrepancies and inequalities in COVID-19 morbidity and mortality have been reported, and if the correct data is collected it may be possible to separate the early life elements that have made people particularly vulnerable to COVID-19 many years later. This will, naturally, then help us identify those that are most at risk from developing the severest forms of COVID-19. In order to do this, we need to recognize socioeconomic and early-life factors as genuine medically and clinically relevant data that urgently need to be collected. Finally, many biological samples have been collected in the ongoing studies. The mechanisms linking the early life environment with a defined later-life phenotype are starting to be elucidated, and perhaps hold the key to understanding inequalities and differences in the severity of COVID-19.

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"It is your reaction to adversity, not the adversity itself that determines how your life's story will develop."

– Dieter F. Uchtdorf –