

1 **The stress hormone cortisol induces virulence in the oral microbiome**

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14 **ABSTRACT**

15 The human microbiome maintains under normal conditions a state of homeostasis. Disruption of
16 homeostasis also referred to as dysbiosis, has been shown to be causally linked to the development of a
17 variety of host diseases. These dysbiotic microbial communities exhibit synergistic interactions for
18 enhanced protection from host defenses, nutrient acquisition, and persistence in an inflammatory
19 environment. One factor that has been shown to lead to dysbiosis of the microbiome is exposure to
20 psychological stressors. Throughout evolution microorganisms of the human microbiome have
21 developed systems for sensing host-associated signals such as hormones associated with those
22 stressors, enabling them to recognize essential changes in its environment thus changing its expression
23 gene profile to fit the needs of the new environment. Cortisol or hydrocortisone is the primary hormone
24 responsible for the stress response, and its levels increase in saliva and serum with the severity of
25 periodontal disease. Most studies have focused on the effect of hormones on growth or virulence of
26 individual organisms. Here we present the impact that cortisol had on the community-wide
27 transcriptome of the oral community. We used a metatranscriptomic approach to obtain first insights
28 into the metabolic changes induced by this stress hormone as well as which members of the oral
29 microbiome respond to the presence of cortisol in the environment.

30

31 **IMPORTANCE**

32 Imbalances of the microbiome also referred to as microbial dysbiosis, lead to a series of different
33 oral diseases. One fundamental question to be answered regarding the pathogenesis of polymicrobial
34 diseases is what are the molecular mechanisms that lead to dysbiosis. The most widely accepted theory
35 to explain the ability of hormones to influence the course of infection involves the suppression of the
36 immune system. Commensal microbiota is involved in stressor-induced immunomodulation, but other
37 biological effects are not yet known. These results bring new insights into the importance of stress

38 hormones such as cortisol as a signal used by the oral microbiome in periodontal disease. Our findings
39 suggest that cortisol can induce virulence of the oral microbiome directly and that Fusobacteria respond
40 rapidly to changes in concentrations of this stress hormone.

41 In recent years a considerable effort has been placed on characterizing the different microbial
42 communities colonizing the human body (1). However, the nature of host-microbial interactions in the
43 microbiome that allow for the maintenance of a stable microbiota is still poorly understood. Among the
44 environmental factors that may alter the equilibrium in host-microbiome homeostasis, host-stress is a
45 known risk factor for a variety of diseases. In case of acute stress, stress response may prepare the
46 immune system for challenges such as infection, but when it becomes chronic, it may influence
47 inflammatory processes leading to the development of systemic or local diseases such as rheumatoid
48 arthritis (2), diabetes (3), or periodontitis (4). Furthermore, physiological stress can also alter the
49 composition of the commensal microbiota in the human microbiome (5).

50 The most widely accepted theory to explain the ability of hormones to influence the course of
51 infection involves the suppression of the immune system. According to this model, stress can activate
52 the central nervous system and the hypothalamus releases corticotropin-releasing hormone and arginine
53 vasopressin that stimulates the release of adrenocorticotropin from the pituitary, which in turn results in
54 the production of cortisol by the adrenal cortex. Glucocorticoids, including cortisol, depress immunity
55 by inhibiting the production of secretory immunoglobulins, and neutrophil functions, all of which may
56 impair defense against infection by periodontal microorganisms (6). However, almost immediately
57 following its first use, cases of adrenaline-associated sepsis were reported (7). It was demonstrated that
58 the dose of *Clostridium* needed to cause infection was significantly smaller when was injected in the
59 presence of a therapeutic level of adrenaline (8). Since then, there have been reports associating the
60 levels of neuroendocrine hormones, such as adrenaline, with infectious diseases, suggesting organisms
61 themselves directly respond to the presence of stress hormones. The study of these interactions has
62 been termed 'microbial endocrinology' (9, 10). Microorganisms that have evolved systems for sensing
63 host-associated signals such as hormones would have an evolutionary advantage over those that have
64 not. Detecting such signals enables the microbiome to recognize essential changes in its environment

65 thus changing its expression gene profile to fit the needs of the new environment.

66 Although most investigations of stress hormones induction of growth and virulence have been
67 carried out with gut-associated bacteria, a few studies have shown that stress hormones have a
68 significant effect on the growth of periodontal pathogens (11, 12). Cortisol or hydrocortisone is the
69 primary hormone responsible for the stress response, and its levels increase in saliva and serum with
70 the severity of periodontal disease (13, 14) Here we present the effect that cortisol had on the
71 community-wide transcriptome of the oral community. We used a metatranscriptomic approach to
72 obtain first insights into the metabolic changes induced by this stress hormone as well as which
73 members of the oral microbiome respond to the presence of cortisol in the environment.

74 Our first experiments consisted of treating samples of oral biofilm with cortisol at a concentration
75 found in the saliva of patients with periodontitis (13, 15). After only 2 hours of incubation in the
76 presence of cortisol, we proceed to perform the analysis, avoiding possible changes related to the
77 growth of individual members of the microbiome and not to the presence of the hormone itself (see
78 Materials and Methods). We assigned the phylogenetic origin of those sequences using Kraken, and
79 phylogenetic profiles were used to identify significant differences between active communities under
80 the different conditions studied by performing linear discriminant analysis (LDA) effect size (LEfSe).
81 Among all the organisms in the oral community, members of the phylum Fusobacteria (class
82 Fusobacteriia and order Fusobacteriales) were significantly more active after the addition of cortisol
83 (Fig. 1). One species, *Leptotrichia goodfellowii*, was substantially more active (Fig. 1b). Species
84 belonging to the *Fusobacteriales*, such as *Fusobacterium nucleatum* have been associated with a wide
85 variety of human diseases, other than periodontitis, including adverse pregnancy outcome, GI disorders
86 (e.g., colorectal cancer, inflammatory bowel disease), cardiovascular disease, rheumatoid arthritis and
87 respiratory tract infections (16). *Leptotrichia* species are typically part of the commensal flora in the
88 oral cavity and genitourinary tract and are seldom found in clinically significant specimens. However,

89 *Leptotrichia* has been found to be in higher proportion in gingivitis (17, 18)

90 We then looked at how cortisol influenced the profiles of expression of the oral microbiome. To
91 this end, we performed enrichment of Gene Ontology (GO) terms analysis, and we observed that, after
92 only 2 hours of exposure to the hormone, the profiles of activities of the whole community were similar
93 to what we previously found in periodontitis progression (19, 20). Interestingly, GO terms linked to
94 host immune response were also over-represented when cortisol was present (Fig. 2a). These results
95 agree with the observation that people with periodontal disease present higher levels of cortisol in the
96 gingival crevicular fluid (13), a serum exudate in direct contact with the oral microbiome. As
97 previously indicated potassium ion transport was significantly under-represented when cortisol was
98 added (Fig. 2b), mimicking ours *in vivo* observations of periodontitis progression (21). In a follow-up
99 manuscript, we demonstrated that ion potassium is a signal in functional dysbiosis of periodontal
100 disease (22). Interestingly, when we look at the activities associated with putative virulence factors, we
101 found a similar pattern of over-represented GO terms related to the addition of cortisol (Fig. S1).
102 Moreover, the more significant fraction of up-regulated putative virulence factors seems to be
103 synthesized by members of the genus *Streptococcus* (Fig. S2). We observed similar results in our two
104 previous studies on periodontitis progression (21) and effect of ion potassium in functional dysbiosis of
105 the oral microbiome (22). As a whole, these results seem to indicate that the presence of cortisol leads
106 to a community-wide response very similar to the one observed *in vivo* during periodontitis.

107 We then performed transcriptome analysis of the effect of cortisol on organisms that we saw were
108 more active in the presence of cortisol (Fig. 1): *Leptotrichia goodfellowii* and *Fusobacterium*
109 *nucleatum*, which is a representative of the order Fusobacteriales and an essential member of the oral
110 microbiome. In just 2 hours of exposure, there was a shift in their transcriptome profiles. *F. nucleatum*
111 showed an increase in biological processes GO terms associated with proteolysis, cobalamin
112 biosynthesis and iron transport (Fig. S3a), which we have previously found associated with the

113 progression of periodontal disease (19, 20). In the case of *L. goodfellowii*, we also found activities
114 related to iron ion transport (Fig. S3b). The intersection of metabolic events that were commonly
115 altered in *F. nucleatum* and *L. goodfellowii* was associated with the growth of the organisms such as
116 lipid A biosynthesis, DNA replication or translation (Fig. S3), which indicates activation of their
117 metabolism. These results are in agreement with the effects observed for the whole oral microbiome
118 where members of the Fusobacteriales order are more active when cortisol was added to the medium,
119 (Fig. 1). Likewise, the intersection of common molecular functions enriched with the addition of
120 cortisol in *F. nucleatum* and *L. goodfellowii* include iron acquisition (iron-ion binding and iron-ion
121 transmembrane transporter activity) and peptidase activities (serine-type endopeptidase activity) (Fig.
122 S4).

123 Altogether, these results show that exposure to cortisol in the oral microbiome increases its
124 virulence, mimicking previous observations on periodontitis *in vivo*. Our results also highlight the
125 importance of *Fusobacteria* and *Leptotrichia* as the first members of the community to respond to the
126 increase of cortisol in the environment.

127
128 **Data Availability.** The sequence datasets used in these analyses were deposited at the Human Oral
129 Microbiome Database (HOMD) under the submission number 20180522
130 (ftp://homd.org/publication_data/20180522/).

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132 **ACKNOWLEDGMENTS**

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134 the National Institutes of Health (NIDCR/NIH) under award number DE021553 and DE021553-05A1.

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136 **Figure Legends**

137 **Figure 1. Statistical differences in the phylogenetic composition of active communities.**

138 Metatranscriptome hit counts were obtained using Kraken against an oral microbiome database. Counts
139 were then analyzed using LEfSe to identify significant differences at the species level between the
140 microbial communities compared. a) Taxonomic representation of statistically and biologically
141 consistent differences between controls without cortisol [green] vs. samples where cortisol was added
142 [red]. b) Histogram of the LDA scores computed for features differentially abundant between controls
143 without cortisol [green] vs. samples where cortisol was added [red].

144

145 **Figure 2. GO enrichment analysis comparing oral microbiome response to the presence and**

146 **absence of added cortisol to the medium.** Enriched terms obtained using GSeq were summarized
147 and visualized as a scatter plot using REVIGO. Only GO terms with FDR adjusted p-value < 0.05 in
148 the 'GSeq' analysis were used. A) Summarized GO terms related to biological processes after addition
149 of cortisol. B) Summarized GO terms related to biological processes with no cortisol added. Circle size
150 is proportional to the frequency of the GO terms; color indicates the log₁₀ p-value (red higher, blue
151 lower). The distance between circles represents GO terms' semantic similarities. Each of the circles
152 represents a GO term, which depending on the similarity in the terms included in them they will be
153 closer or more distant in the graph.

154 **Supporting Information Legends**

155 **Figure S1. Community-wide GO enrichment analysis of differentially expressed virulence factors**
156 **in response to the presence and absence of added cortisol to the medium.** Putative virulence factors
157 were identified by alignment of the protein sequences from the different genomes against the Virulence
158 Factors Database (VFDB) as described in the methods section. Enriched terms obtained using GOseq
159 were summarized and visualized as a scatter plot using REVIGO. Only GO terms with FDR adjusted p-
160 value < 0.05 in the 'GOseq' analysis were used. A) Summarized GO terms related to biological
161 processes after addition of cortisol. B) Summarized GO terms related to biological processes with no
162 cortisol added. Circle size is proportional to the frequency of the GO terms; color indicates the log₁₀ p-
163 value (red higher, blue lower). The distance between circles represents GO terms' semantic similarities.
164 Each of the circles represents a GO term, which depending on the similarity in the terms included in
165 them they will be closer or more distant in the graph. In red are activities we have previously seen
166 associated with periodontitis (19, 21).

167

168 **Figure S2. Ranked species by the number of up-regulated putative virulence factors in the**
169 **metatranscriptome.** Putative virulence factors were identified by alignment of the protein sequences
170 from the different genomes against the Virulence Factors Database (VFDB) as described in the
171 methods section. Numbers in the graph refer to the percentage of hits for the different species for the
172 putative virulence factors identified. We selected only species whose percentage of putative virulence
173 factors from the total of the community was higher than 1%. We included also results from 2 previous
174 studies one on periodontal disease progression (21) and another where we showed that potassium was a
175 crucial signal in dysbiosis (22). In red, species that were ranked at the top of the three studies.

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178 **Figure S3. Biological processes GO terms associated with changes in gene expression profiles in**
179 ***Fusobacterium nucleatum* and *Leptotrichia goodfellowii*.** GO terms were assigned to differentially
180 expressed genes due to the addition of cortisol and summarized using REVIGO. a) GO terms
181 associated with up-regulated genes in *F. nucleatum* b) GO terms associated with down-regulated genes
182 *L. goodfellowii*. In green are metabolic activities that were associated with up-regulated genes in both
183 *F. nucleatum* and *L. goodfellowii*. In red are activities we have previously seen associated with
184 periodontitis (19, 21).

185

186 **Figure S4. Common molecular function GO terms associated with changes in gene expression**
187 **profiles in *Fusobacterium nucleatum* and *Leptotrichia goodfellowii*.** GO terms were assigned to
188 differentially expressed genes due to the addition of cortisol and summarized using REVIGO. Networks
189 of over-represented molecular functions from *F. nucleatum* and *L. goodfellowii* were then uploaded to
190 Cytoscape, and the intersection of the two networks was extracted and plotted as shown in the figure.
191 In red are activities we have previously seen associated with periodontitis (1, 22).

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- Cortisol
+ Cortisol

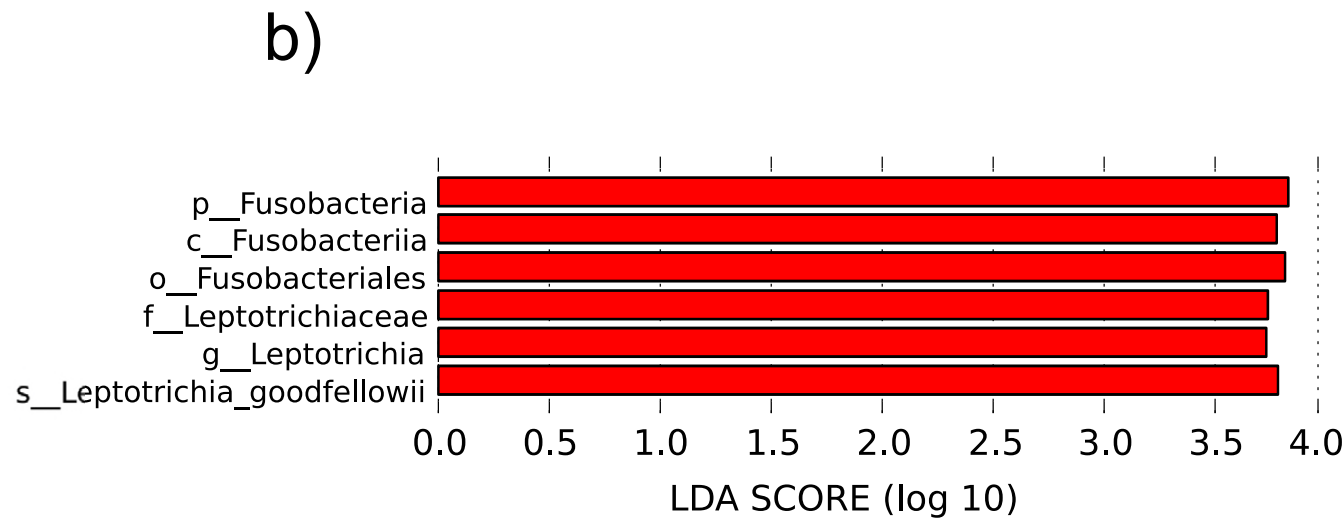
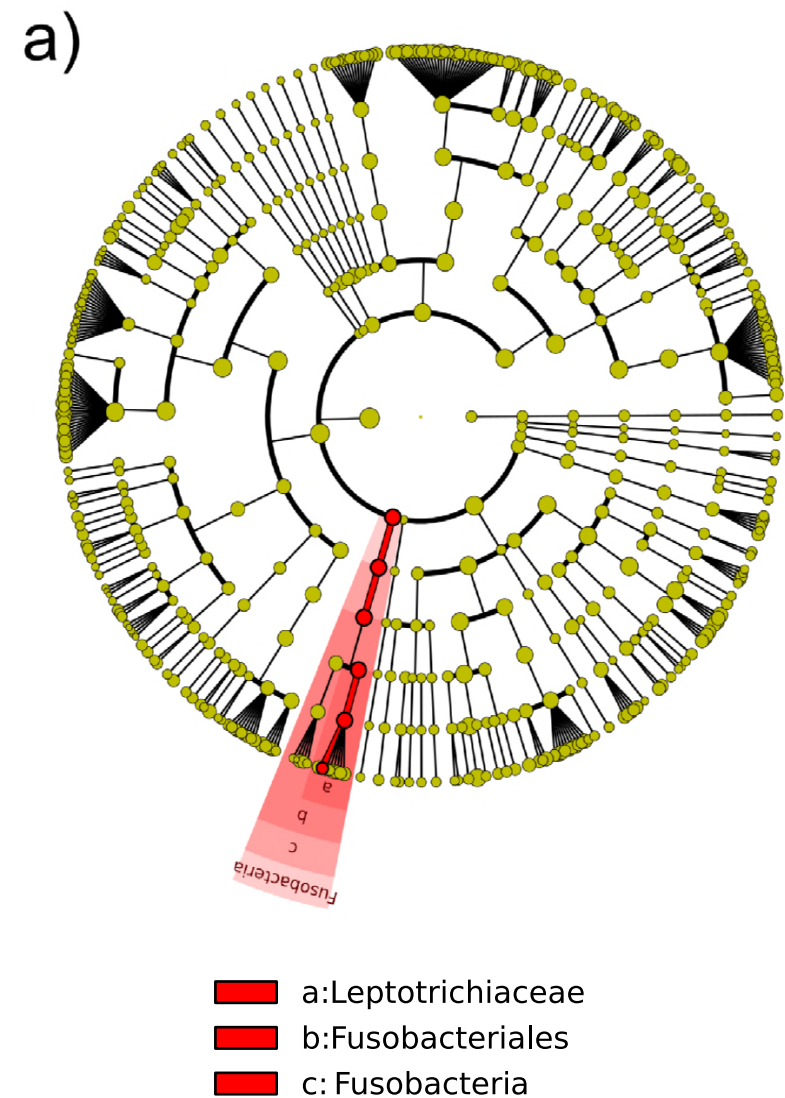


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a) Taxonomic representation of statistically and biologically consistent differences between controls without cortisol [green] vs. samples where cortisol was added [red].

b) Histogram of the LDA scores computed for features differentially abundant between controls without cortisol [green] vs. samples where cortisol was added [red].

