

Proteomic investigations of in vitro and in vivo models of periodontal disease

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1 Proteomics Clinical Applications

2 Review manuscript

3 Title: Proteomic investigations of *in vitro* and *in vivo* models of periodontal disease

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24 Abstract:

25 Proteomics has currently been a developing field in periodontal diseases to obtain protein
26 information of certain samples. Periodontal disease is an inflammatory disorder that attacks the
27 teeth, connective tissues and alveolar bone within the oral cavity. Proteomics information could
28 provide proteins that are differentially expressed in diseased or healthy samples. This review
29 provides insight into approaches researching single species, multi species, bacteria, non-human and
30 human models of periodontal disease for proteomics information. The approaches that have been
31 taken include gel electrophoresis and qualitative and quantitative mass spectrometry. This review
32 was carried out by extracting information about *in vitro* and *in vivo* studies of proteomics in models
33 of periodontal diseases that have been carried out in the past two decades. The research has
34 concentrated on a relatively small but well known group of microorganisms. A wide range of models
35 has been reviewed and conclusions across the breadth of these studies are presented in this review.

36 Text:

37 Introduction

38 Periodontal diseases are highly prevalent in humans and other mammals. There is a continuum of
39 severity from mild reversible gingivitis to severe periodontitis that causes irreversible damage to the
40 hard and soft tissues and may results in exfoliation of teeth in untreated sufferers. Gingivitis is the
41 reversible inflammation of the gingival tissues and affects an estimated 50-100% of adults [1] . The
42 severest forms of periodontitis affect 10% of the population, is the 6th most prevalent disease
43 worldwide [2], and are linked with systemic diseases such as diabetes [3], rheumatoid arthritis [4]
44 and chronic kidney disease [5].

45 Periodontal disease is initiated by accumulation of bacteria within the mouth giving rise to gingivitis,
46 defined by gingival inflammation. The plaque biofilm has been extensively studied by culturing and
47 non-culturing methods. One of the models of bacterial species associated with different levels of
48 clinical inflammation in the mouth was created by Socransky et al [6]. These authors clustered
49 bacteria, via statistical analysis, associated with clinical phenotypes together and this gave rise to a
50 number of colour coded complexes. The red complex contains three bacteria, *Porphyromonas*
51 *gingivalis*, *Treponema denticola* and *Tanarella forsythia*, associated with the most destructive
52 periodontal disease. *Streptococcal* species were, mostly, clustered together in the yellow complex
53 and bridging species such as *Fusobacterium nucleatum* in the orange complex. The yellow complex
54 was rarely associated with periodontal disease and the orange complex was associated with deep
55 periodontal pockets. This is an extremely brief overview and interested readers can access greater
56 depth of information from reviews such as [7].

57 In individuals susceptible to periodontitis, long term retention of the plaque biofilm adjacent to the
58 gingivae allows for colonisation and expansion of microbial biofilms incorporating bacteria that are
59 more destructive to the host tissues. This forms the basis for the transition to periodontitis, which is
60 irreversible. The host immune response to biofilms is one of the key factors in the development of
61 periodontitis; for example patients with periodontitis have over reactive polymorphonuclear
62 leukocytes (PMNLs) that produce antibacterial responses [8, 9] that also cause bystander damage
63 and destruction of host tissues, including the epithelial and bone structures. The nuances of host
64 genetics and phenotype influencing disease development and progression are being studied [10], as
65 well as the contribution of periodontal plaque biofilms [11]. However, as can be expected, both the
66 human and the bacterial contributions are highly variable and whilst, *ex vivo* samples can reveal
67 many aspects, often the sample size or variability between donors can make identification of

68 pathways difficult without prior knowledge or a way of stratifying donors. Thus development of
69 model systems to understand either the host or bacterial responses to changes in the oral
70 environment or complex changes in more consistent experimental models of periodontal disease are
71 required for deeper understanding. Use of proteomic methods allows for a non-presumptive
72 approach to understanding and exploring the role proteins, ~~the work horses of cells and organisms,~~
73 play in periodontal disease. This review aims to present the data published over the last two decades
74 exploring models of periodontal disease by using proteomics. Figure 1 shows an overview of the
75 approaches taken.

76 In the proteomic investigation of periodontal disease various techniques have been used: the most
77 popular are two dimensional polyacrylamide gel electrophoresis (2D PAGE) followed by mass
78 spectrometry, often matrix assisted laser desorption ionisation mass spectrometry (MALDI MS), and
79 liquid chromatography tandem mass spectrometry (LC-MS/MS) or shotgun proteomics. These two
80 techniques have been the mainstays of proteomic discoveries for at least the last two decades and
81 the development and refinement of mass spectrometry and chemical labelling techniques has meant
82 that in more recent times more proteins can be quantitatively identified by LC-MS/MS.

83 2D PAGE relies on the separation of proteins in two dimensions, most commonly by charge and mass
84 by isoelectric focusing and sodium dodecyl sulphate (SDS) PAGE. These creates a two dimensional
85 map of protein spots. These spots are ranged across the pH spectrum mostly between pH 3-10 and
86 most densely between pH 4-7. *In silico* representations of the proteome on 2D PAGE plots often
87 show a lack of proteins with a pH around pH7.5 as these proteins would likely precipitate in cellular
88 buffer environments. The protein spots can represent individual proteins, though this depends on
89 the resolution and size of the gel used, or multiple proteins in one location with similar properties.
90 Multiple spots may also be identified as the same parent protein but may have distinct properties,
91 known or unknown, such as post translational modifications, splice variants or isoforms.
92 Quantification of these different properties can be revealing of underlying pathologies [12] or
93 cellular changes [13]. It should be noted that one dimensional PAGE may also provide similar insights
94 as 2D PAGE but with lower resolution. Either technique has often relied on use of image analysis
95 software to quantify and compare proteins patterns to identify proteins of interest. Development in
96 these imaging techniques allow for warping of protein patterns within individual gels and
97 sophisticated approaches to protein detection can be used to pick up lower abundance proteins.
98 These can then be excised from the gel and tryptically digested for identification by mass
99 spectrometry. The earliest studies (eg [14]) almost exclusively used MALDI MS to identify mass
100 fingerprints, however changes in instrumentation have led to the use of tandem mass spectrometry

101 and the sequencing of proteins of interest, giving a higher identification reliability. For example in
102 early studies multiple spots may have been picked for identification but not all protein identification
103 (eg [14]) may have been realised, whereas this is more likely now.

104 One of the methods for profiling complex biological samples is using liquid chromatography tandem
105 mass spectrometry (LC-MS/MS). Samples of proteins of interest are digested with proteolytic
106 enzymes, such as but not limited to trypsin. These peptides are then separated by high performance
107 liquid chromatography (HPLC) for molecular fractionation couple to the mass spectrometer. The LC
108 technique is most often reverse phase and capable of analysing small and large molecules of various
109 polarities [15]. Other chromatographies may also be used, particularly if a two dimensional LC
110 approach is adopted: the use of basic reverse phase [16] has gained in popularity recently as it
111 decreases the amount of processing that might be associated with high salt strategies such as ion
112 exchange chromatography.

113 With LC-MS/MS techniques it is possible to quantitatively assess amounts of proteins isolated from
114 samples of interest. Mass spectrometry is not innately quantitative, because of the variability in
115 tryptic peptide properties which results in variability between mass spectrometric runs and that only
116 sample a small percentage of the total peptides in a sample are examined in the mass spectrometer.

117 Relative quantitation can be divided into labelled and ~~labelled~~ free techniques. Within the labelled
118 techniques Stable Isotope Labelling with Amino acids in Cell culture (SILAC) utilises combinations of
119 isotope labelled arginine and lysine to give rise to three different masses that can be detected within
120 a mass spectrometry instrument. It is a metabolic labelling technique. SILAC requires the uptake of
121 these amino acids so is useful for model systems such as eukaryotic or prokaryotic cell culture but
122 can also be used with whole organisms, such as flies and mice. The advantage of SILAC is that every
123 sample of interest is labelled before any kind of treatment occurs at the protein level, eg gingival
124 fibroblasts would be labelled prior to challenge with oral pathogens, and this means that errors
125 associated with processing samples are minimised within the work flow. As none of the papers
126 included in this review used SILAC interested readers are referred to [17] for further details. Other
127 labelling techniques label at the peptide level, such that proteins need to be digested for each
128 sample separately before labelling, which may allow for some variation to be incorporated into the
129 samples. Thus it is very important to ensure standard operating procedures are followed to minimise
130 risk of this occurring. While SILAC is limited to three channels to compare with the peptide labelling
131 techniques, at time of writing, up to 11 channels are available routinely, though combinations of
132 techniques have been reported to expand this number through hyperplexing [18]. This larger
133 number allows for comparison of more samples, experimental conditions or replicates of individual

134 conditions within one experiment, which may reduce variability. Some of the labelling techniques
135 include tandem mass tags (TMT), isobaric tags for relative and absolute quantitation (iTRAQ), ¹⁶O/¹⁸O
136 labelling, isotope-coded affinity tag (ICAT).

137 Label free techniques have the advantage of having no limit on the number of samples or
138 experimental conditions that can be compared. However they rely on the performance of the liquid
139 chromatography system and/or the mass spectrometer to accurately identify the peptide and its
140 quantity. Software advances have made this a main stream analysis route; although it is not without
141 its challenges. With any of the techniques described so far it is not always possible to identify
142 reproducibly peptides within a sample. The development of sequential window acquisition of all
143 theoretical mass spectra (SWATH MS)[19] complements the techniques touched on above as it aims
144 to identify all peptides within a sample, giving greater coverage and reproducibility. The technique is
145 described as data independent-acquisition (DIA), as opposed to data dependent acquisition (DDA),
146 as the identification of the peptides is divorced from the quantitation by the creation of a library of
147 all expected peptides before analysis of experimental samples. Whilst of great interest this
148 technique has not yet been used with periodontal models. SWATH MS combines the advantages of
149 shotgun, such as high throughput, with those of selected reaction monitoring (SRM), such as high
150 reproducibility and consistency. SRM or multiple reaction monitoring (MRM) are examples of
151 absolute quantitation (AQUA) that require a targeted approach rather than the discovery
152 approaches mentioned above.

153 Each of the techniques, 2D PAGE or shotgun MS techniques will identify proteins of interest to the
154 research. High quality mass spectra can generate highly accurate protein identifications; however
155 there is a desire to confirm these discoveries. Validation studies may take the form of SRM
156 experiments, Western blotting, immunohistochemistry, enzyme linked immunosorbent assays
157 (ELISAs) or other techniques. These techniques often allow for the examination of one or more
158 proteins at a greater level of detail through expansion of the experimental conditions, for example at
159 multiple time points or in greater number of samples. These kinds of data can add further evidence
160 and insight into proteomics discoveries.

161 Experimental models often allow for larger quantities of starting material than, for comparison,
162 clinical samples. This means that greater in depth analysis may be possible. In the case of 2D PAGE
163 larger gels may be able to be used with Coomassie Blue instead of silver staining. These two changes
164 may mean that identification of proteins of interest is more likely. With shotgun techniques larger
165 samples may mean that two dimension techniques can be used increasing the number of samples
166 run through the mass spectrometer and thereby reducing the number of peptides in each duty cycle.

167 This may allow for greater numbers and thereby coverage of proteins and or proteomes being
168 analysed.

169 Post translational modifications are a profound force within cellular systems that can alter protein
170 properties, for example cellular location and activation status of enzymes. Proteomic tools allow for
171 analysis of these changes to proteins either directly by identification of the modifications in the mass
172 spectrometer or via prefractionation of modified proteins. Of general interest modification of
173 proteins by phosphorylation has given rise to the wide spread analysis of phosphoproteomics and
174 signalling cascades. Other modifications such as oxidation [13] and glutathionylation [20] have been
175 of interest in the field of oral models of periodontal disease, though many other modification have
176 been explored in other areas [21].

177

178 The word proteomics was coined in 1994 [22] and there have been many changes in the technology
179 and analysis of proteomes since. One such development is the idea that not just one proteome is
180 examined within a sample and this gives rise to the idea of metaproteomics: the study of proteins
181 from multiple organisms. Metaproteomics is of particular interest in examining the complex
182 microbial communities that inhabit many parts of the Earth – from the communities in water waste
183 treatment plants [23], biogas plants [24], to in the human gut [25] and mouth [26]. Metaproteomics
184 brings its own unique challenges: generation of comprehensive databases to search against, how to
185 handle homologous peptides found across organisms, taxonomic and functional evaluation, and
186 computational handling of huge datasets. Software packages have been developed in the past
187 decade to help with some of these challenges (reviewed by [27]); there are many choices and most
188 are accessible but as Heyer et al [27] note their use may be restricted to a small number of scientific
189 groups due to lack of bioinformatic skills and funding for some. For the development of databases
190 and to decrease computational burden tailoring the database to the sample from which the
191 metaproteome is derived is key. These tailored databases can be created from metagenomes or
192 when the taxonomic composition is known, for example with *in vitro* created multispecies biofilms
193 from compiled genomes. Interestingly, metaproteomic searches against multiple metagenome
194 sequences from the same sample can improve discovery of proteins as many metagenome
195 sequences do not comprehensively represent the microbial communities from which they are
196 sampled [28]. Homologous proteins can be shared across a number of species in the metaproteome
197 and assignment of these proteins may not be possible to a single species. For example, Rudney *et al.*
198 [29] identified 357 unique peptides from a metaproteomic analysis of salivary microbiota but were
199 only able to assign 11% at the species level. Thus the taxonomic value of these proteins can be

200 estimated using the lowest common ancestor (LCA), particularly where unique peptides are absent,
201 which can be very common in complex datasets [30]. Lastly, metaproteomics studies often identify
202 5-30% of the spectra in a dataset. A further 30% of spectra are derived from solvent and background
203 components [31] and this leaves at least another 30% that are unidentified. Further development of
204 software may assist in unveiling these spectra.

205 *In vitro* studies

206 *In vitro* studies using simplified models of the oral cavity start with explorations of single species and
207 move towards complex model systems incorporating multiple cells types and organisms. Each type
208 of model can answer some questions about the pathogenesis of periodontal disease and proteomic
209 approaches can verify that the results are broad. All the publications discussed below are
210 summarised briefly in Table 1.

211

212 Single species models

213 In the simplest models single bacterial species have been examined to understand how changes in
214 their environment affect the proteome. Zilm et al [14] examined the effect of pH on the growth of
215 *Fusobacterium nucleatum*. This species is highly adaptable and is often called a bridging organism for
216 its ability to change the biofilm and allow for growth of more fastidious species. In respect to pH, the
217 gingival pocket may commence as an acidic environment but metabolism of GCF nutrients can
218 neutralise this pH [32] and this allows for growth of species such as *Porphyromonas gingivalis*,
219 which in turn can contribute to a further shift to an alkaline environment after fermentation of
220 amino acids [33]. Zilm et al [14] used 2D PAGE to illustrate how changes in pH alter the proteome of
221 *F.nucleatum* subspecies *polymorphum*. They discovered that acidic conditions (pH6.4) led to reduced
222 cell numbers but up-regulation of the 2-oxoglutarate pathway suggesting requirement of energy-
223 expending protective mechanisms; in contrast alkaline conditions (pH 7.8) caused greatest changes
224 in the glycolytic pathway, iron limitation and upregulation of enzymes that would contribute to
225 maintenance of a more alkaline environment.

226 Steeves et al [34] utilised *F. nucleatum* subspecies *nucleatum* to determine the bacterial response to
227 changing oxidative stress conditions as *F. nucleatum* has been shown to have a protective role
228 against reactive oxygen species (such as hydrogen peroxide, H₂O₂). Utilising multiple 2D PAGE gels
229 these authors explored both the duration of exposure to H₂O₂ (0-7h) and the concentration of H₂O₂
230 ~~hydrogen peroxide~~ (0-0.3mM) to which the cultures were exposed. The analysis revealed that the
231 AhpC/thioredoxin system played a prominent role in the response to oxidative stress. The proteins

232 of the system were upregulated and modified. This is of particular note because the 2D PAGE
233 revealed differences between in the isoforms of AhpC that revealed differences in the oxidation
234 state of cysteine residues, such that it was likely due to the hyperoxidation of these residues to
235 sulfinic or sulfonic acids. Hyperoxidation like this can cause irreversible oxidation whilst the
236 upregulation of the proteins may also combat this by sequestration of oxidative species.

237 *F. nucleatum* is also known to be associated with production of hydrogen sulphide (H₂S) [35] [36]
238 which contributes to halitosis in periodontal disease patients [37, 38]. Basic et al [13] evaluated a
239 range *Fusobacterium* species and strains for H₂S production and then focused on more in depth
240 studies to identify the proteins and substrates involved. The research highlighted a wide range of
241 proteins involved and conditions required for their expression.

242 In the *in vitro* exploration of single bacterial species that are of interest in the oral cavity there has
243 been a range of publications on *Aggregatibacter actinomycetemcomitans* (previously
244 *Actinobacillus actinomycetemcomitans*) examining the role the bacteria have in production of the
245 immune response which is integral to the destructive phenotype of aggressive periodontitis. Whilst
246 these publications do not necessarily describe a model for periodontal disease they do highlight the
247 role of proteomics, and particularly immuno proteomics in the discovery of underlying contributors
248 to periodontal disease. Paul-Satyaseela et al [39], Rylev et al [40] and Kieselbach et al [41] have used
249 different *A. actinomycetemcomitans* strains and preparations to explore the immunoreactivity of
250 sera derived from patients with aggressive periodontitis. These approaches have identified proteins
251 from outer membrane proteins, whole cell extracts and outer membrane vesicles, respectively, that
252 are immuno-reactive. This work highlights that there are multiple strains and preparation techniques
253 that can illustrate protein discovery for further disease mechanism studies.

254

255 Multi species models

256 Several groups have investigated the interaction of multiple species important for oral disease in the
257 same experiment. Zainal-Abidin et al [42] explored the proteome of red complex (*P. gingivalis*, *T.*
258 *denticola* and *T. forsythia*) species after 90h in flow cell culture. Extensive validation using
259 fluorescence *in situ* hybridisation (FISH) of the conditions revealed that *P. gingivalis* and *T. denticola*
260 dominated biofilm growth in this model system, which was adopted because of the requirement of
261 the removal of planktonic cells and the formation of a genuine biofilm, which cannot be achieved
262 with static growth conditions. The proteomic analysis used differential oxygen labelling to compare
263 planktonic and biofilm cultures. This allowed for quantitation of proteins from *P. gingivalis* and

264 *T.denticola* (Table 1), which suggested that *P. gingivalis* changed iron acquisition strategy in the
265 biofilm. The switch was from the use of HmuY/R to HusA/B reflecting a competition for hemin with
266 the other species present as HusA has a greater affinity for hemin compared to HmuY [43, 44].
267 However, there were only two replicates used in these experiments and the reproducibility within
268 these was not always good for some proteins and therefore Western blotting was used to verify the
269 iron acquisition changes.

270 A series of reports on the interaction between *P. gingivalis* [45], *S. gordonii* [46] and *F. nucleatum*
271 [47] were published over the course of five years by the same group. *P. gingivalis*, when introduced
272 into the mouths of human volunteers localises to streptococcal rich plaque [48] and in supragingival
273 areas *F. nucleatum* will help to scavenge oxygen allowing for *P. gingivalis* growth [49]. Thus the
274 authors postulated that it is likely that there will be three species communities of these
275 microorganisms *in vivo*. Confocal microscopy was used to illustrate the multi species communities
276 generated. Each species was compared when prepared on its own to when prepared with the other
277 two species in an 18h low exogenous nutrient model, mimicking common periods of low nutrient
278 availability in the oral cavity, which started with equal numbers of the three bacteria when in mixed
279 culture. This allowed for probing of the adaptation of each species to the multispecies community
280 environment. For *P. gingivalis* [45] 1156 proteins were detected in the community, 403 were up
281 regulated and 89 were down regulated ; for *S. gordonii* [46] 649 proteins were detected in the
282 community, 163 increased and 174 decreased; and for *F. nucleatum* [47] 1135 proteins were
283 detected in the multispecies community with 109 increased and 430 decreased. Overall all the
284 species demonstrated wide ranging adaptations to their proteomes when in multispecies biofilms.
285 Comparisons were also made between dual species biofilms as well, which illustrated the *S. gordonii*
286 had a more dominant response to the presence of *P. gingivalis* rather than *F.nucleatum* [46]. Each
287 paper describes in detail the changes in the pathways of the bacteria examined, some highlights are
288 detailed here. *P. gingivalis* showed a reduction in the abundance of 40 cell envelope proteins
289 indicating a substantial change in this location; *P. gingivalis* showed changes in thiamine biosynthesis
290 which was likely due to adaptation to nutrient transfer or cross-feeding [50]. The authors also
291 investigated the role of HmuR by generating a mutant lacking HmuR. This altered strain was
292 deficient in community formation and the authors speculated that this was unlikely to be associated
293 with hemin uptake and more likely to be associated with biofilm formation though a cohesive
294 function and adhesin properties. *S. gordonii* demonstrated decreases in the phosphoenolpyruvate
295 dependent phosphotransferase (PTS) sugar transport system that indicated high levels of sugar and
296 low pH in the mixed species community; additionally *S. gordonii* showed a shift in by-product
297 formation, away from ethanol but towards lactate, such that it likely provided nutrients for the other

298 species; and whilst detection of known adhesins was inconsistent there was a trend for a decrease in
299 this class of protein by *S. gordonii* in mixed cultures [46]. *F. nucleatum* showed a more complex
300 response to growth in dual and multi species communities [47]; it was less able to compete with the
301 other species and each species (in the dual model) elicited a different response to that with the
302 multispecies model. Concentrating on the multi-species model the overall effect on amino acid
303 fermentation was not clear however a shift from lactate towards butanoate as by-products fits with
304 the production of lactate by *S. gordonii* reported earlier. As seen in the other species there was also
305 a trend for some adhesins to be decreased in expression in the multispecies communities. All three
306 studies reported lower DNA repair proteins and additionally *F. nucleatum* appeared to have
307 increases in antioxidant proteins. Following on from these complex studies the authors have
308 published further exploration, including an investigation in to the adaptation of streptococcal 4-
309 aminobenzoate/*para*-amino benzoic acid (p-ABA) on the *P. gingivalis* proteome [50] which reported
310 the upregulation of FimA and Mfa1 and fimbrial accessory proteins resulting in increased adherence
311 to gingival cells; an increase in gingipain protease activity; and haemin uptake proteins. Overall this
312 suggested that *P. gingivalis* uses p-ABA as an adaptation signal to change in the rich subgingival
313 environment.

314 Moving into even more complex multispecies communities ten and eleven species biofilms grown
315 over 64h culture have been examined by Bao et al [51, 52]. These are true metaproteomic studies
316 utilising specially compiled databases for protein identification. This model is substantially more
317 complex and potentially more similar to the human oral cavity in that the ten species are grown on a
318 hydroxyapatite disc, mimicking the subgingival tooth surface: indeed the initial starting conditions
319 require saliva and serum along with growth media and the discs are incubated in anaerobic
320 conditions. In these two papers the effect of the introduction of an eleventh bacterial species is
321 assessed by proteomics: the species introduced are either *A. actinomycetemcomitans*, associated
322 with aggressive periodontitis, or *Anaeroglobus geminatus*, a relatively newly discovered species
323 associated with chronic periodontitis [53] and apical periodontitis [54]. Comparing the abundance of
324 proteins identified from each species across the two papers there are quite marked differences in
325 the quantities of proteins from each species: with *Campylobacter rectus*, for example, revealing very
326 few proteins overall in the publication comparing the ten species biofilm with and without *A.*
327 *geminatus* but one of the highest quantities in the publication comparing the ten species biofilm
328 with and without *A. actinomycetemcomitans*. This is reflected in some way by the quantity of this
329 species in the two studies; however *C. rectus* is not one of the dominant players of *F. nucleatum*, *P.*
330 *intermedia*, *S. anginosus*, *S. oralis* or *V. dispar*. The inherent variability in this model may help reveal
331 more about *in vivo* biofilms and how they may change between sites within one oral cavity due to

332 association with neighbouring species and nutrient availability as each biofilm in this model has the
333 same starting material but is allowed to mature for a lengthy period (64h). The authors also show
334 [51], by FISH, the localisation of *A. actinomycetemcomitans* in clusters within the matured biofilm
335 supporting this idea. The authors point out that some species, *Actinomyces* oris, *T. denticola* and *T.*
336 *forsythia*, have very few proteins identified and that this may also be due to underrepresentation in
337 the corresponding databases, which has been acknowledged as a complication of metaproteomics.
338 These studies also identified universal biofilm shifts in numerous molecular functions, biological
339 processes and cellular component gene ontologies though it was not feasible to attribute these
340 changes to individual species due to the nature of the comparisons. The major shifts were in
341 metabolic processes and iron transport, perhaps indicating the manoeuvring of the individual
342 species into the multispecies community.

343

344 Bacterial and host models

345 The next step in creating an *in vitro* model system is to incorporate host cells and examine the
346 response of the host cells and/or the microorganisms. Bostanci et al [55] examined the effect on the
347 proteins secreted, or secretome, of organotypic three dimensional gingival epithelial tissues when
348 the ten species biofilm mentioned above, as well as a seven species model which did not contain the
349 red complex bacteria, was suspended 1mm above the epithelial tissue. After 24h the ten species
350 biofilm, in comparison to unstimulated epithelial tissue, had induced an innate inflammatory
351 response including chemokine production and various leukocyte activation processes and proteins
352 such as the S100A8 and S100A9 complexes, whereas tissue development and cytoskeletal process
353 appeared to decrease which could be associated with a disruption of the tissue integrity; by 48h
354 many processes designated as negatively regulated were increased and responses to stress and
355 wound healing were decreased. These processes could be seen as the repercussions of earlier
356 disruption to tissue integrity and apoptotic cascades. There was also a comparison between the ten
357 and seven species biofilm stimulated secretomes to investigate the role the three red complex
358 bacteria might have. Overall the presence of the three red complex bacteria seemed to interfere
359 with epithelial cell growth and metabolism but also decrease processes of blood coagulation and
360 haemostasis. This may, *in vivo*, be associated with the bleeding phenotype for which these bacteria
361 were clustered together.

362 Taking the research a step further the same group [26] also published a study examining the effect
363 of the eleven species biofilm (including *A. actinomycetemcomitans*) when co-cultured in a perfusion

364 bioreactor for 24h. The host cells in this system were immortalized gingival epithelial cells (HGEK-
365 16), immortalized gingival fibroblasts (GFB-16) and a monocytic cell line (Mono-Mac-6) perfused
366 through a three dimensional collagen sponge. Proteomic analysis was performed on the culture
367 supernatants of the co-culture and the human and bacterial cells alone. In addition analysis of
368 biofilm lysates with and without co-culturing with human cells was performed. As before, the
369 secretome human proteins demonstrated a profile associated with the induction of an inflammatory
370 response particularly through the induction of Toll-like receptor processes. The bacterial
371 contribution to the secretome was diverse and overall there appeared to be a larger number of gene
372 ontologies that were down regulated rather than up regulated over the 24h incubation. Examination
373 of the biofilms themselves in this co-culture had previously revealed that most of the bacterial
374 species were suppressed in the presence of host tissue [56]. However bioinformatic analysis
375 suggested that overall the bacteria, when in the presence of the tissue, had an increase in gene
376 ontologies associated with movement, particularly with cytokinesis proteins and development of
377 thicker cell walls which would make them fitter for mobility. Overall the use of this model is
378 elucidating early response of gingival tissue to periodontal like biofilms and allowing for greater
379 understanding of the initiation of periodontal inflammation.

380 Another study has examined the role of *P. gingivalis* lipopolysaccharide (LPS) on human gingival
381 fibroblasts (HGF) [57] at both the proteome and secretome levels. *P. gingivalis* LPS is heterogeneous,
382 containing numerous lipid A moieties and presenting two predominant isoforms PgLPS₁₆₉₀, which is
383 penta-acylated, and PgLPS_{1435/1449}, which is tetra-acylated. The effect of these two isoforms on HGF
384 were used in comparison to *E.coli* LPS: PgLPS₁₆₉₀ upregulated inflammatory proteins while
385 PgLPS_{1435/1449} increased anti-inflammatory proteins in cell extracts and both caused increases in
386 proteins of the antioxidant response, such as peroxiredoxin, thioredoxin and superoxide dismutase.
387 Whereas analysis of the secretome showed an increase in inflammatory molecules, proteases and
388 extracellular matrix components.

389 The responses of differentiated THP-1 cell line, monocyte-macrophage like cells, to live *P. gingivalis*,
390 heterogeneous PgLPS or Pg fimbrial protein FimA, were explored by Saba et al [58]. In this 2D PAGE
391 analysis 32 protein spots were picked from gels and a number of overlapping identifications made.
392 The proteins were generally highly abundant, for example actin and enolase. The stimulations with
393 isolated PgLPS and PgFimA resulted in similar protein profiles when compared to untreated cells,
394 however the PgFimA stimulations did not reach statistically different changes leading the authors to
395 conclude that PgFimA is not involved in critical upregulation of the inflammatory response. As
396 technology and quantitative techniques have changed substantially since this publication it would be

397 interesting to revisit this type of experiment to try to tease out some of the effects on the less
398 abundant proteins.

399 Lastly in the *in vitro* model systems Chen et al [59] examined the effect of *A.*
400 *actinomycetemcomitans* cytolethal distending toxin (CDT) on the Jurkat T cell line. *A.*
401 *actinomycetemcomitans* CDT is known to induce apoptosis of T cells and this publication sought to
402 identify which pathway was involved by using a collection of point mutations in CDT. The authors
403 homed in on 17 apoptosis related genes that were quantified across the cell extracts from Jurkat
404 cells treated with CDT wildtype or the different mutants. Effector caspase-3 was induced by both
405 wildtype and mutants but the initiator caspases were not captured in the proteomic analysis.
406 However, western blotting was used to verify that caspase 8 was activated in cells treated with
407 wildtype CDT but not with mutant CDTs. The proteomic analysis did identify the SUMO-conjugating
408 enzyme UBC9, which catalyses the addition of ubiquitin like protein SUMO to proteins. Sumoylation
409 of p53, also identified in the unmodified state in the data set as being upregulated, has been
410 implicated in DNA damage, a downstream point in apoptosis, which is in line with other reports [60].

411

412 *In vivo* studies

413 *In vivo* studies harness the whole body response of periodontal inflammation to explore questions
414 about periodontal disease induction, progression, natural history or resolution. Different mammals
415 can give insights into these factors though may involve oral species that are naturally present in the
416 mouths of the experimental animals as well as those of particular interest for human disease.
417 Perhaps uniquely with periodontal disease it is also possible to understand the initiating processes
418 by using human volunteers through the experimental gingivitis model. The studies detailed below
419 are listed briefly in Table 1.

420

421 Non-human models

422 Rodents have often been used in the exploration of periodontal disease. In terms of proteomic
423 examination Yang et al [61] considered the response of first molar mandibles in periapical
424 periodontitis by comparing molars that were exposed to *E.coli* LPS and the oral cavity to unaffected
425 contralateral molars. Only a handful (7) proteins were up regulated whereas 150 were down
426 regulated after the two weeks of induced periapical periodontitis. The authors confirmed hexokinase
427 induction by Western blotting: hexokinase is the initial enzyme in glycolysis but is also an activator of

428 NLRP3 inflammasome and apoptosis. However the conclusion that this may be a biomarker of
429 periapical periodontitis would require many subsequent studies.

430 One aspect of human periodontal disease that would be difficult to follow is the natural progression
431 from health to gingivitis to periodontitis due to the varied time it takes to progress, if at all, and
432 lifestyle mediated factors. Thus following a model of this has a distinct advantage to understand in
433 greater detail the continuum of periodontal disease. Davis et al [62] examined such progression in a
434 canine model where pet dogs were allowed to progress from health through gingivitis to the start of
435 periodontitis by removal of any mechanisms of plaque removal over the course of 60 weeks. Ten
436 teeth in eight individual dogs were selected from a larger study and gingival crevicular fluid
437 proteome was monitored. There was a wide variation between the types of teeth but a core of 84
438 proteins were detected in all samples: in general across the progression of disease protein quantity
439 increased potentially in line with GCF volume, though this was not measured, however using
440 univariate mixed model analysis it was possible to identify haptoglobin, haemoglobin, S100A8 & A12,
441 fibrinogen and 14-3-3 beta/alpha as significantly increased in periodontitis. These signify the change
442 in inflammation and potentially bleeding on probing used to define this disease stage.

443 The cynomolgus monkey (*Macaca fascicularis*) is an old world monkey highly related to humans,
444 sharing 92.5% of the human genome [63]. It is one of the most important non-human primate
445 animal models in biomedical research and has been used to understand the effect of complement 3
446 (C3) inhibitor Cp40 in reducing periodontal ~~measurements~~destruction, such as probing pocket depth
447 and clinical attachment loss, in monkeys with established periodontitis [64]. As with the canine
448 model reported by Davies et al [62] there was considerable variation in the number of proteins
449 among individual macaques and in this study between time points. Gene ontology analysis of the
450 GCF proteins revealed immune system process to be the most abundant biological process, and
451 within this category macrophage activation was highly abundant. In terms of pathways, the
452 alternative and classical complement pathways were highly enriched compared to the genome
453 background, indicating their importance to natural periodontitis. In response to treatment with the
454 C3 inhibitor 124 proteins were down regulated including tetranectin, mannose binding lectin and
455 vitronectin, indicating along with other results that Cp40 mediates inhibition via the alternative
456 pathway of complement activation.

457

458 Human models

459 There have been two proteomic studies [65, 66] examining the induction and resolution of human
460 experimental gingivitis in gingival crevicular fluid. Both studies utilised 21 days for induction taking
461 samples at baseline (0), 7, 14 and 21 days and a slight variation in exploration of resolution: Grant et
462 al [65] used samples solely at 35 days (14 days after resumption of oral hygiene measures) and
463 Bostanci et al [66] used samples from days 25, 30 and 35 to examine resolution associated changes
464 in the GCF proteome. Grant et al [65] used the same ten donors for all samples whereas Bostanci et
465 al [66] used ten donors to examine gingivitis induction and a different ten donors for gingivitis
466 resolution. Both groups demonstrated increases in gingival index and GCF volume associated with
467 gingivitis showing that the experimental induction of gingivitis was successful. In the examination of
468 the proteomes both groups also attempted to discover non-human proteins and small numbers
469 were reported in each study, however the human protein identification dominated. Hierarchical
470 clustering was used in both studies to find novel proteins of interest: Grant et al [65] identified
471 proteins that followed the pattern of clinical markers and included proteins indicative of ribbon
472 synapses and primary cilia; Bostanci et al [66] highlighted the discovery of metallothionein-2 and
473 ubiquilin-4 as small intracellular, metal-binding cysteine rich antioxidant proteins during induction of
474 gingivitis and down regulation of neutrophil derived antibacterial proteins, such as myeloperoxidase,
475 in the resolution arm of the study. The different experimental approaches to quantitation (iTRAQ
476 labels in Grant et al and label free quantitation in Bostanci et al) highlighted different proteomes:
477 Grant et al could discover proteins that changed in all the time points (186 human) but didn't look at
478 proteins only found in some of the samples; whereas Bostanci et al could discover more proteins
479 overall (254 human) but only 5 in all of the samples. This highlights not only the highly variable
480 nature of the donors and the stochastic nature of mass spectrometry but also the contribution of the
481 variety of proteins found in gingival crevicular fluid: this fluid is made up of serum proteins, tissue
482 exudate and microorganism proteins and cellular lysates. This mix will yield a very dynamic range of
483 protein abundances and protein types. It is widely acknowledged that some saliva proteins are not
484 readily detectable by trypsin directed database searches [67] and this may also be true for other
485 compartments.

486

487 Summary

488 A wide variety of approaches have been taken in the creation of models of periodontal disease to
489 explore this complex disease. Simple single species models have delved in depth into the changes
490 that individual species undergo during transition that can be encountered in the mouth; multispecies
491 systems have been used to look at 3-11 species in culture comparing to individual species and to

492 insertion of extra species; the response of host cells to bacteria either alone, as purified parts of
493 single bacteria or to multispecies biofilms has been explored; and finally whole organism models
494 have been employed – from rats to humans. Groups publishing more than one paper have been
495 consistent in their approaches but between groups changes in times of incubation, proteomic
496 approaches and bioinformatics explanations can be found. This could make the evaluation of such a
497 field very complex, however there are consistent messages in how biofilms adapt to different
498 microorganisms and how host cells respond to biofilms. At present there has been focussed research
499 on a relatively small but well known group of microorganisms, which makes sense in respect to the
500 restrictions of metaproteomics. As the more is known about more recently discovered oral flora
501 there may be more that can be gleaned from the proteomic approach.

502 Conflict of Interest Statement:

503 The authors have declared no conflict of interest.

504

505 Table 1. List of publications examined in the compilation of this review. Papers were selected from a
506 search of Pubmed using 'oral' and 'proteom*' or 'mass spectrometry' and then filtered for
507 relevance.

508

509

510 Figure 1. Summary of the approaches taken to explore models of periodontal disease. Diagram
511 prepared with Biorender.

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