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

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

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Abstract:

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

Text:

Introduction

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

In vitro studies

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

Single species models

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

Steeves et al [34] utilised *F. nucleatum* subspecies *nucleatum* to determine the bacterial response to changing oxidative stress conditions as *F. nucleatum* has been shown to have a protective role against reactive oxygen species (such as hydrogen peroxide, H_2O_2). Utilising multiple 2D PAGE gels these authors explored both the duration of exposure to H_2O_2 (0-7h) and the concentration of H_2O_2 ~~hydrogen peroxide~~ (0-0.3mM) to which the cultures were exposed. The analysis revealed that the AhpC/thioredoxin system played a prominent role in the response to oxidative stress. The proteins

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

F. nucleatum is also known to be associated with production of hydrogen sulphide (H_2S) [35] [36] which contributes to halitosis in periodontal disease patients [37, 38]. Basic et al [13] evaluated a range *Fusobacterium* species and strains for H_2S production and then focused on more in depth studies to identify the proteins and substrates involved. The research highlighted a wide range of proteins involved and conditions required for their expression.

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

Multi species models

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

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

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

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

Bacterial and host models

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

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

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

Another study has examined the role of *P. gingivalis* lipopolysaccharide (LPS) on human gingival fibroblasts (HGF) [57] at both the proteome and secretome levels. *P. gingivalis* LPS is heterogeneous, containing numerous lipid A moieties and presenting two predominant isoforms $PgLPS_{1690}$, which is penta-acylated, and $PgLPS_{1435/1449}$, which is tetra-acylated. The effect of these two isoforms on HGF were used in comparison to *E.coli* LPS: $PgLPS_{1690}$ upregulated inflammatory proteins while $PgLPS_{1435/1449}$ increased anti-inflammatory proteins in cell extracts and both caused increases in proteins of the antioxidant response, such as peroxiredoxin, thioredoxin and superoxide dismutase. Whereas analysis of the secretome showed an increase in inflammatory molecules, proteases and extracellular matrix components.

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

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

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

In vivo studies

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

Non-human models

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

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

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

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

Human models

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

Summary

A wide variety of approaches have been taken in the creation of models of periodontal disease to explore this complex disease. Simple single species models have delved in depth into the changes that individual species undergo during transition that can be encountered in the mouth; multispecies 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.

Conflict of Interest Statement:

The authors have declared no conflict of interest.

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.

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

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