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1 A novel microbiological medium for the growth of periodontitis associated pathogens

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7 Highlights

- 8 • A novel growth medium for culturing periodontal pathogens
- 9 • More representative of the periodontal environment
- 10 • Cultivates a selection of periodontal pathogens and can be used with human cell culture

11 Abstract

12 A novel microbiological medium designed to be more representative of gingival crevicular fluid. Chosen
13 representative periodontal microorganisms showed good growth with minimal effect on human cell
14 viability. This will enable more comparisons between different periodontitis associated organisms and
15 their potential role in host health and systemic disease.

16 Keywords: Chronic Periodontitis, Growth Medium, Oral Microbiology, Cell Culture

17 Chronic Periodontitis (CP), an infection of the periodontium, leading to the formation of
18 periodontal pockets and tooth loss if untreated (1). It is the most common infectious inflammatory
19 disease worldwide affecting humans (2) with 45 % of English adults having at least one periodontal
20 pocket (3). CP is of particular interest as it can be detrimental to the health of the patient and a risk factor
21 for myriad diseases such as Alzheimer's, (4) cardiovascular disease (5) and diabetes mellitus (6).
22 Subgingival bacteria are the main aetiological agents behind the inflammatory processes seen in CP and
23 induce dysregulation of the normal oral microbiota (7) and an increased flow of gingival crevicular fluid
24 (GCF), an inflammatory exudate that bathes the gingival crevice and favours the growth of anaerobic
25 periodontal microorganisms (8). A key contributor to CP is *Porphyromonas gingivalis* (9), however, it
26 appears that no singular group of organism is responsible for causing CP and it is likely that polymicrobial
27 synergy and dysbiosis of multiple periodontitis associated pathogens play a role in modulation of the
28 disease (10). Many of these periodontitis associated pathogens are difficult to culture *in vitro*, which is
29 partly compounded by the lack of available broad host growth media. Periodontal pathogens are usually
30 fastidious anaerobes and rely on nutrients secreted by other bacteria during synergism along with specific
31 microbial and non-microbial nutrient sources (11). The aim of this study was to develop a growth medium
32 that can facilitate the culture of a wide selection of periodontitis associated microorganisms and enable
33 further study of potential systemic disease links via tissue culture based investigations. With the growth
34 requirements of many of these organisms being specifically evolved to the unique environment of the
35 gingival crevice, emulating components of the GCF can allow a developed medium to be more
36 representative of this environmental interface and allow more representative growth of periodontal
37 microorganisms.

38 Bacterial strains were maintained in growth media as recommended by the manufacturer.
39 *Actinomyces israelii* (NCTC 12972), *Campylobacter showae* (NCTC 12843), *Fusobacterium nucleatum*
40 *subsp. Fusiforme* (11326) and *Prevotella intermedia* (NCTC 13070) were cultivated in anaerobic conditions
41 (80 % N₂ - 10 % H₂ - 10 % CO₂) in a Baker-Ruskin Concept Plus anaerobic incubator (Ruskin, UK) at 37°C for
42 48 h. *P. gingivalis* (NCTC 11834) was cultivated in an anaerobic incubator for 72 h. *Rothia dentocariosa*
43 (10917), *Streptococcus constellatus* (NCTC 11325) and *Streptococcus sanguinis* (NCTC 7863) were
44 cultivated in carbon rich conditions (5 % CO₂) in a CO₂ incubator (LEEC, UK) at 37 °C for 48 h.

45 The base medium utilised was Basal Medium Mucin (BMM) as it has successfully been shown to emulate
46 saliva for the growth of dental plaque biofilms in an 'artificial mouth' system (12). The constituents of
47 BMM contained: 2.5 g/L partially purified pig gastric mucin, 10 g/L proteose peptone, 5 g/L trypticase

48 peptone, 5 g/L yeast extract, 2.5 g/L KCl, 1 mMol/L urea, 1 mMol/L arginine, 1 mg/L of haemin and 1 mg/L
49 of menadione, all purchased from Sigma Aldrich, UK.

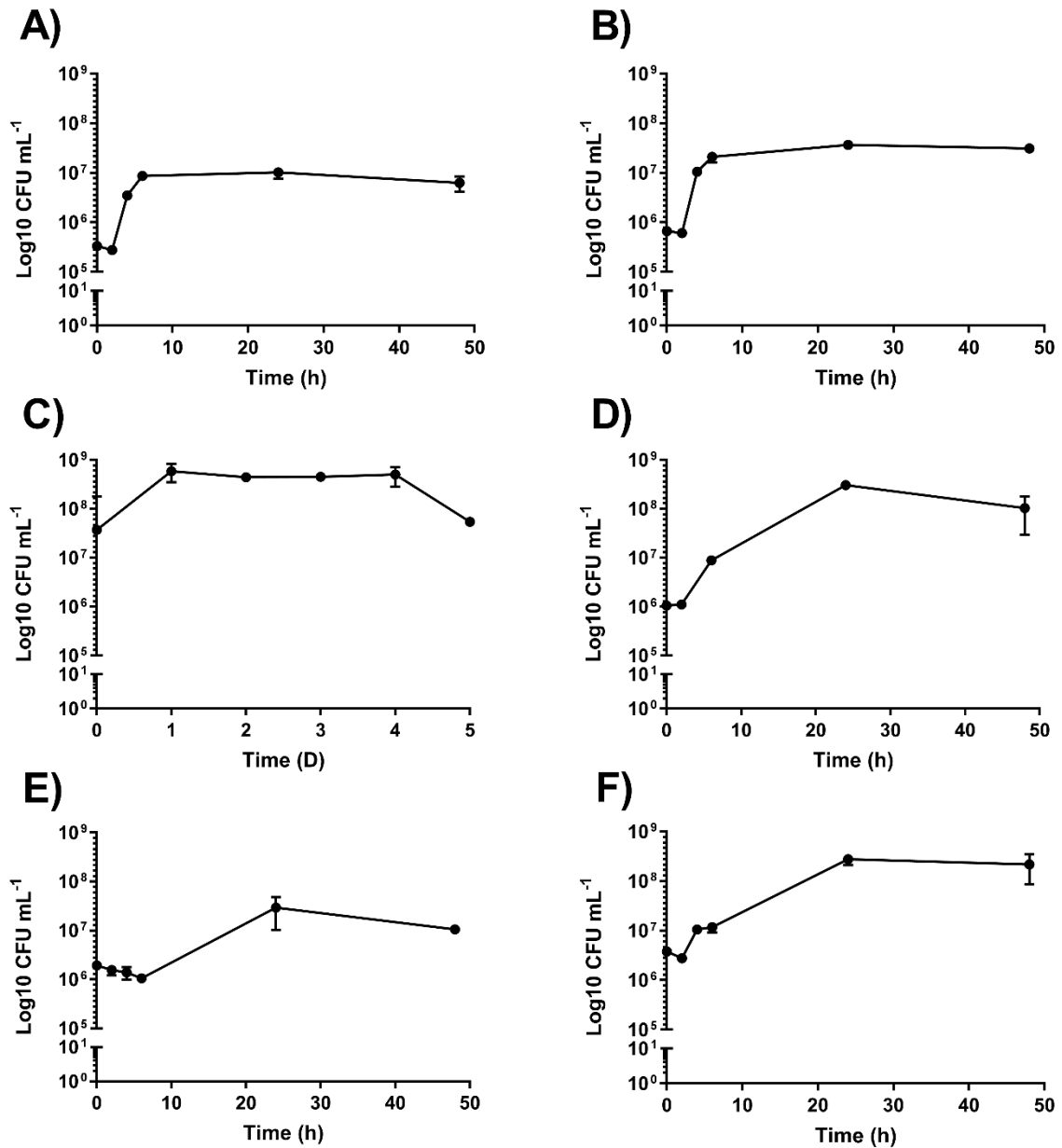
50 The new growth medium, Artificial Gingival Crevicular Fluid – Periodontal (AGCF-P) was composed of
51 BMM with multiple modifications. From BMM, 1 mg/mL of haemin and menadione were omitted and 5
52 % horse blood (TCS Biosciences) was added once cooled to 50 °C after sterilisation by autoclave. This was
53 designed to emulate the inorganic and organic constituents of GCF which are notably similar to plasma,
54 as infiltration and degradation of red blood cells occurring during CP (13). The pH of the medium was
55 adjusted to reflect the actual pH of GCF during CP (7.96 ± 0.1) (14). Buffering of pH was performed using
56 the phosphate buffer system of Potassium Phosphate monobasic anhydrous and Sodium Phosphate
57 dibasic heptahydrate. Cysteine was added as a reducing agent to further mimic the reducing capacity and
58 support the growth of anaerobic organisms. The full composition of AGCF-P was: 10 g/L proteose
59 peptone, 5 g/L trypticase peptone, 5 g/L yeast extract, 2.5 g/L KCl, 2.5 g/L partially purified pig gastric
60 mucin, 0.5 g/L L-cysteine, 0.17 g/L L-arginine, 0.18 g/L L-urea, 1.68 g/L Potassium Phosphate monobasic
61 anhydrous, 41.31 g/L Sodium Phosphate dibasic heptahydrate and 5 % horse blood. If creating solid
62 media 12 g/L of agar no 3 was added. All reagents were procured from Sigma Aldrich, UK with the
63 exception of trypticase peptone (Scientific Laboratory Supplies, UK) and horse blood.

64 The growth of the bacterial strains on AGCF-P agar was determined by streaking a single isolated colony
65 onto an AGCF-P agar plate. This plate was sub-cultured again to ensure continuity and photographed (not
66 shown). Growth of bacterial strains in liquid culture was tested by inoculating a single colony into 5 mL
67 of AGCF-P broth and grown for the NCTC recommended duration; growth was demonstrated by
68 inoculating an AGCF-P agar plate and further incubation. For growth curves, three overnight cultures of
69 the chosen microorganism were adjusted to an absorbance of 1.0 ± 0.05 at 540 nm obtained using a
70 spectrophotometer (Jenway, UK). One millilitre per 100 mL of culture was inoculated into three sterile
71 culture flasks with liquid AGCF-P growth media equilibrated overnight. Growth was assessed at 0, 2, 4, 6,
72 24, and 48 h or daily for 5 day for *P. gingivalis* and at each time point colony forming units per mL
73 (CFU/mL) were quantified using the Miles-Misra droplet method and viability was determined (15).

74 Human Gingival Fibroblast (HGF-1) primary cells (LGC standards, UK) were cultured in Dulbecco Modified
75 Eagle Medium (DMEM) with Glutamine and Glucose (Lonza, UK), supplemented with 10 % foetal calf
76 serum (Lonza, UK), 50 µg/mL streptomycin (Lonza, UK) and 50 µg/mL penicillin (Lonza, UK). Immortalised
77 Human Kidney Proximal Tubule cells (HK-2) (ATCC, UK) were cultured in 50:50 glucose free DMEM
78 (Invitrogen, UK) and Hams F-12 medium (Lonza, UK) supplemented with 10 % foetal calf serum (Lonza,
79 UK), 50 µg/mL streptomycin (Lonza, UK), 50 µg/mL penicillin (Lonza, UK) and 2.5 mMol glutamine (Lonza,
80 UK). Cells were growth-arrested in serum free cell culture media, in the absence of foetal calf serum.

81 To assess the effect of AGCF-P bacterial growth medium on mammalian cell viability, the AGCF-P medium
82 was incubated for 5 days in anaerobic conditions without bacterial inoculation. At 0, 1, 2, 3, 4 and 5 days
83 aliquots of the AGCF-P medium were plated out to check for bacterial contamination. Viability testing
84 and statistical analysis was conducted as detailed in (16).

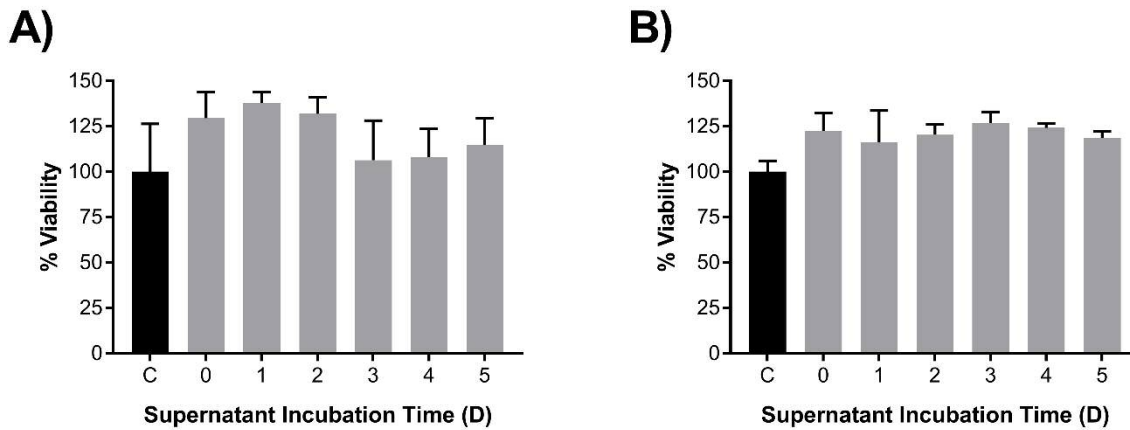
85 The growth of periodontal associated pathogens was assessed in liquid and solid AGCF-P media
86 by inoculation or streaking with a single colony of the chosen organisms. *A. israelii*, *C. showae*, *F.*
87 *nucleatum* subsp. *Fusifforme*, *P. intermedia*, *P. gingivalis*, *R. dentocariosa*, *S. constellatus* and *S. sanguinis*
88 all showed good growth in liquid and solid cultures even with multiple subcultures (data not shown).
89 Growth curves of representative examples of periodontal pathogens were conducted in AGCF-P broth
90 over 2 or 5 days for *P. gingivalis*, to demonstrate growth (Figure 1). All organisms reached a good level of
91 growth after 24 h, averaging at 10^7 CFU/mL indicating that the media was sufficiently nutritious to
92 support the representative selection of periodontal microorganisms.



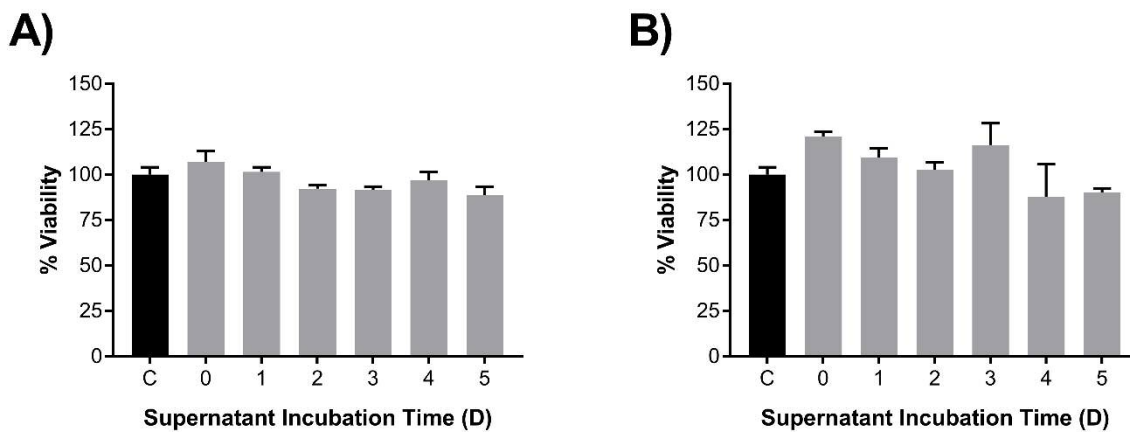
93
 94 **Figure 1.** Growth curves of periodontitis associated pathogens cultured in AGCF-P broth. A) *Actinomyces israelii* B)
 95 *Fusobacterium nucleatum* C) *Parvimonas micra* D) *Porphyromonas gingivalis* E) *Streptococcus constellatus* F) *Streptococcus*
 96 *sanguinis* (n=3).

97 The effect of the AGCF-P medium on the cell viability of HGF-1 (Figure 2), and HK-2 (Figure 3) was assessed
 98 by exposing the cells to 1:20 dilutions of AGCF-P supernatants incubated over a 5-day period in anaerobic
 99 conditions. Although the cell viability of HGF-1 was slightly increased (Figure 2), there was no significant
 100 effect of AGCF-P supernatants on the viability of these cells or HK-2 cells after 24 h (Figure 2A and 3A) or
 101 48 h (Figure 2B and 3B) of incubation, as compared to control cells exposed to serum-free cell culture
 102 medium only. The mild positive effect of AGCF-P on the viability of primary gingival fibroblasts can be
 103 attributed to an increased cell proliferation due to nutrients provided by AGCF-P.

104



105
 106 **Figure 2.** Cell viability of HGF-1 cells after 24 h (A) or 48 h (B) of incubation with 1:20 dilutions of AGCF-P supernatants
 107 collected over 5 days. Viability is presented as percentage change from the control (C: cells exposed to serum-free cell culture
 108 medium) and error bars represent SEM (n=3).



109
 110 **Figure 3.** Cell viability of HK-2 cells after 24 h (A) or 48 h (B) of incubation with 1:20 dilutions of AGCF-P supernatants collected
 111 over 5 days. Viability is presented as percentage change from the control (C: cells exposed to serum-free cell culture medium)
 112 and the error bars represent SEM (n=3).

113 Mimicking the chemical components of the physiological environment is a successful strategy
 114 that has been used to cultivate particularly fastidious or uncultivable organisms and also can influence
 115 the bacterial products present (17). Emulating some of the elements of the GCF should allow for more
 116 representative growth of disease-related organisms as alternative nutrient sources available may
 117 stimulate regulatory changes in gene expression profiles leading to the production of environment
 118 specific bacterial products, such as metabolites and secreted proteins (18). AGCF-P has been shown to
 119 cultivate a selection of periodontal organisms and could be used to test their secreted products in human
 120 cells without the growth media confounding the results, facilitating the development of *in vitro* and *ex*
 121 *vivo* models of CP. The production of more biologically relevant conditions furthers research into the
 122 pathogenesis of CP, enabling wide-ranging comparisons between organisms to identify key microbial
 123 products and their effect on the human host.

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 125 commercial, or not-for-profit sectors.

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