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New growth media for oral bacteria

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Abstract

New growth media have been designed for the iron-controlled co-cultures of three oral bacteria. These media share a common core composition enabling the switch from mono- to co-cultures, and efficiently promote both planktonic and biofilm cultures of *Porphyromonas gingivalis*, *Treponema denticola* and *Streptococcus gordonii.*

Keywords: *Porphyromonas gingivalis, Treponema denticola, Streptococcus gordonii,* Periodontitis, Iron, Growth media

Periodontitis is characterized by the damage of periodontal tissues causing periodontal pockets and bone loss, leading to tooth loss. The evolution of the disease is linked to a modification of the oral microbiota (Meuric *et al.*, 2017a). Anaerobic bacteria are closely associated with the transition from healthy to pathological conditions. The virulent switch of the microbiota may be influenced by host-related factors such as systemic iron levels. Indeed, a recent study demonstrated that severe periodontitis was associated with the severity of iron burden in patients with HFE-related hereditary haemochromatosis, an autosomal recessive genetic disease leading to iron overload (Meuric *et al.*, 2017b). To further evaluate the impact of iron concentration on the fitness of the oral microbiota, we developed a simplified experimental model with bacterial species for which interactions are known to influence the evolution of periodontal disease, namely *Streptococcus gordonii* (primary colonizer), *Porphyromonas gingivalis* and *Treponema denticola* (anaerobic

pathogens)(Ng *et al*., 2016). *P. gingivalis* and *T. denticola* are keystone pathogens strongly associated with the severity of the disease (Orth *et al*., 2011). They were frequently found together in deep periodontal pockets (Byrne *et al*., 2009; Tan *et al*., 2014). Both species are anaerobic fastidious organisms and all experiments so far were carried out in complex media such as OMIZ-M (Wyss, 2007), OBGM (Orth *et al*., 2010) and BM (Loo *et al*., 2000). However, to further study the role of iron, it is important to avoid empirical components such as brain heart infusion and yeast extract, which are usually contaminated with high residual concentrations of iron. In this study, new growth media, with only bovine serum albumin (BSA), casamino acids (CAA) and/or glucose as carbon sources, were designed for the individual- or co-cultures of *P. gingivalis*, *T. denticola* and *S. gordonii* (Medium for Mixed Bacterial Community, MMBC) (Table 1).

Table 1. Composition of MMBC media

Medium Type (Iron concentration in µM)*			Composition	
MMBC-3 (0.56 +/-4.30E-03)*	MMBC-1 (0.45 +/- 1.39E-02)*		NaH_2PO_4	5 mM
			KCl	5 mM
			$MgCl_2$. $7H_2O$	5 mM
			Menadione	0.581 µM
			BSA	3675 mg L^{-1}
			CAA	2500 mg L^{-1}
	MMBC-2 (0.50 +/- 5.37E-04)*		Adenine	1.35 mg L^{-1}
			FAD	1 mg L^{-1}
			Folic acid	1 mg L^{-1}
			Pyridoxal phosphate	5 mg L^{-1}
			Fumarate	0.5 g L^{-1}
			Pyruvate	0.55 g L^{-1}
			TPP	25 mg L^{-1}
			Inosine	2.7 mg L^{-1}
			CoA	1 mg L^{-1}
			Volatile fatty acids**	0.001%(v/v) each
			D-Biotin	0.05 µM
			Nicotinic acid	0.04 mM
			D-Glucose	6 g L^{-1}
			$MnSO_4$	10 mg L^{-1}
			L-Arginine-HCl	1 mM
			L-Tryptophan	0.1 mM
			Cysteine- HCl	1.3 mM
			L-Glutamic acid	4 mM

			$(NH_4)_2SO_4$	0.6 g L^{-1}

*Data are the average concentrations of two independent freshly prepared media
** Volatile fatty acids: valeric acid, isovaleric acid and isobutyric acid.
Table 1

A core composition sufficient for the growth of *P. gingivalis* (MMBC-1) was further supplemented with components necessary for the growth of *T. denticola* (MMBC-2) and *S. gordonii* (MMBC-3). The quantification of iron in the MMBC media was performed by Inductively Coupled Plasma Mass Spectrometry (ICP-MS), on X-Series II from Thermo Scientific® equipped with collision cell technology (AEM2 Facility, University of Rennes 1), as previously described (Cavey *et al.*, 2015). All three media contained less than 0.56 µM of iron (Table 1), making them suitable for iron metabolism/transport related studies. These media were first tested for planktonic mono- or cocultures in anaerobic conditions at 37°C.

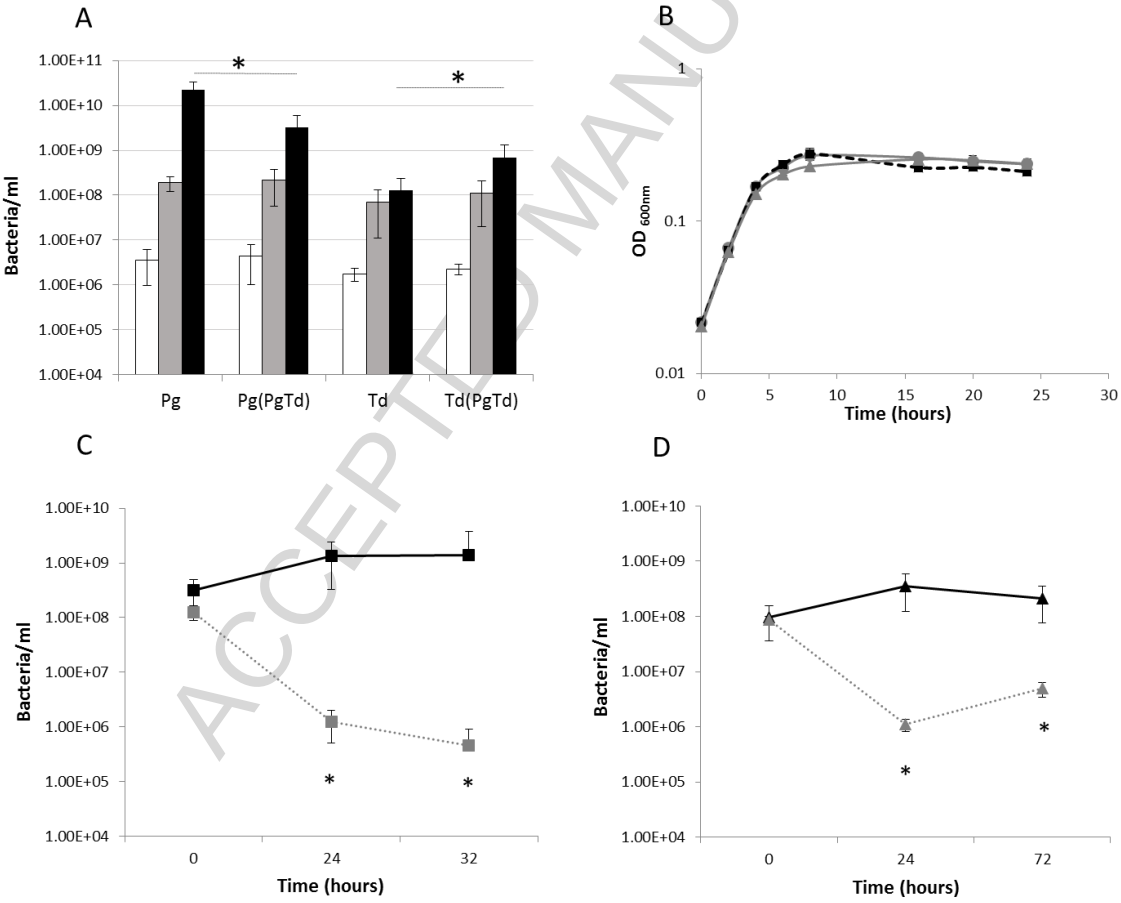


Figure 1. Planktonic cultures

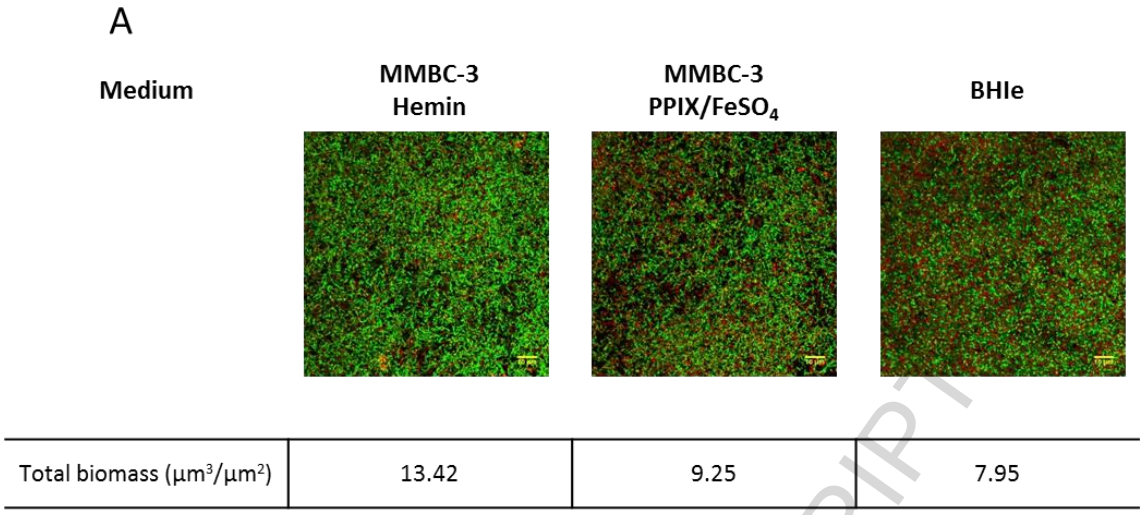
A) Mono- and cocultures of *P. gingivalis* and *T. denticola* were grown in MMBC-3 media with 8 µM of hemin. Quantification of bacterial species in cocultures was carried out by qPCR. Samples were collected at 0 (white bars), 30 (grey bars) and 100 hours (black bars) after anaerobic incubation at 37°C. Pg: monocultures of *P. gingivalis*. Pg(PgTd): quantification of *P. gingivalis* when cocultured with *T. denticola.* Td: monocultures of *T. denticola.* Td(PgTd): quantification of *T. denticola* when cocultured with *P. gingivalis.* Data are the average of triplicate assays. p values were calculated with student's T-test. *p<0.05, **p<0.01.**B)** *S. gordonii* Challis DL1 was grown in MMBC-3 plus 8 µM hemin (grey circle), or 8 µM $FeSO_4$ together with 0.08 µM PPIX (grey triangle) or 8 µM $FeSO_4$ (black square) at 37°C in anaerobic conditions. OD values are the average of 3 experiments. **C)** Growth and survival of *P. gingivalis* and **D)** *T. denticola* were monitored at 37°C, in anaerobic condition, in MMBC-3 with 8 µM hemin, in the presence (grey symbol) or absence (black symbol) of *S. gordonii*. Quantifications of *T. denticola* and *P. gingivalis* in cocultures were carried out by qPCR. Data are the average of 3 experiments performed in triplicates. p values were calculated with student's T-test; *p<0.05, **p<0.01.

Figure 1

Strains of *S. gordonii* Challis DL1 (Chen *et al.*, 2004), *P. gingivalis* TDC60 (Watanabe *et al.*, 2011) and *T. denticola* ATCC35405 (Chan *et al.*, 1993) were used. For monocultures, the growth of each species was measured by optical density at 600 nm over a period of time in MMBC media supplemented with hemin or $FeSO_4$ as iron sources. For cocultures, quantification of each bacterial species at different time points was carried out by qPCR with species-specific 16S rRNA primers as previously described (Ammann *et al.*, 2013; Martin *et al.*, 2017)(Fig. 1). Monocultures of *P. gingivalis* grew similarly in all MMBC media containing hemin or ferrous iron with protoporphyrin IX (PPIX) (Fig. 1A for MMBC-3). 8 µM of hemin was found as the optimal concentration for *P. gingivalis* growth (data not shown). The removal of hemin impaired the growth of *P. gingivalis* and *T. denticola* (data not shown). Without hemin, the growth of *T. denticola* was restored by 8 µM of $FeSO_4$, whereas *P. gingivalis* needed both $FeSO_4$ and protoporphyrin IX (PPIX) (data not shown). The optimal concentration of PPIX that promoted the growth of *P. gingivalis* without impairing the growth of *T. denticola* was defined as 0.08 µM. Moreover, addition of PPIX in $FeSO_4$-containing MMBC-3 was not required for the growth of *S. gordonii* (Fig.1B). The growth of *T. denticola* was enhanced by co-culture with *P. gingivalis* in MMBC-2 (data not shown) and in MMBC-3 (Fig.1A) such as it was previously observed in the rich OBGM medium which contained yeast extract and brain heart infusion as carbon sources (Tan *et al.*, 2014). Interestingly, an antagonistic behaviour was observed in

co-cultures of *S. gordonii* with either *P. gingivalis* (Fig. 1C) or *T. denticol*a (Fig. 1D). The presence of *S. gordonii* impeded the growth and survival of both pathogens.

To assess bacterial growth in sessile conditions, biofilms were grown in sterile µ-slide 8-chambered coverslip (ibiTreat, Ibidi), that were previously coated with 0.22-µm filtered sterile human saliva (25% v/v) collected from at least six healthy volunteers (Martin *et al.*, 2017). Formation of three-species biofilms in anaerobic conditions at 37°C was evaluated in MMBC-3 medium plus hemin or $FeSO_4$ and PPIX, as compared with brain-heart infusion broth (BHIe) (Biomérieux, France) supplemented with menadione (10 µg mL^{-1}) and hemin (5 µg mL^{-1}). At 24 hours, biofilms were stained with 5 µM of Syto®40 nucleic acid dye, a blue fluorescent membrane-permeant stain (Molecular Probes, Lieden, The Netherlands) and 40 µM of Propidium Iodide (Thermo Fisher) diluted in PBS before observation *in situ* with a Leica TCS-SP8 confocal laser scanning microscope (Leica Microsystems, Wezlar, Germany). After imaging, bacteria collected from biofilms were quantified by qPCR as described by Martin *et al.*, (2017).



Total biomass ($\mu m^3/\mu m^2$)	13.42	9.25	7.95

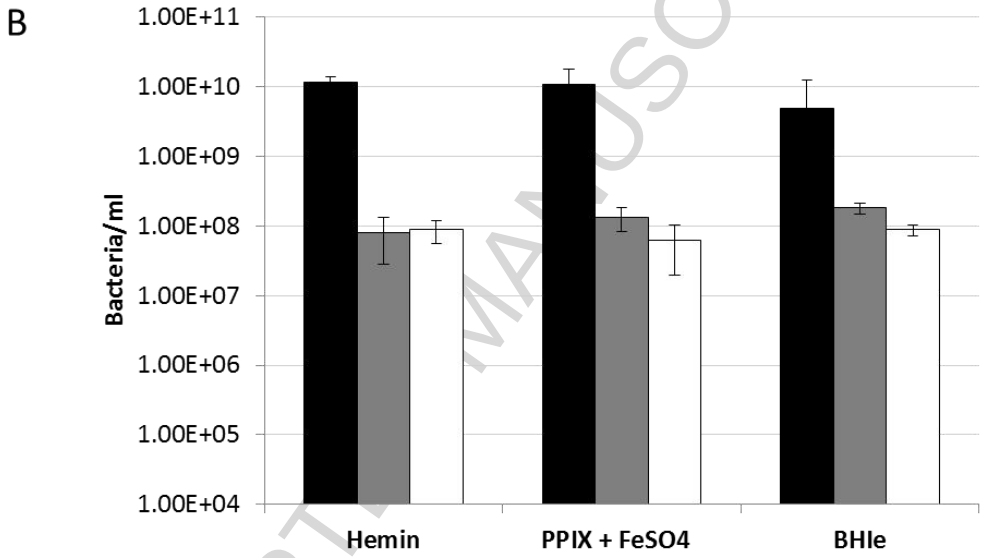


Figure 2. Three-species biofilms in MMBC-3 with different iron sources as compared with BHIe. Biofilms were grown in MMBC-3 medium plus 8 µM hemin or 8 µM $FeSO_4$/0.08 µM PPIX or in BHIe. 24-hours biofilms stained with Syto®40 nucleic acid dye (Live cells-green colour) and Propidium Iodide (Dead cells-red colour). **A).** The figure shows representative images of 3 experiments with the 3D maximum z projection. The biomass ($\mu m^3/\mu m^2$) was estimated using Comstat 2 plugin of ImageJ software V1.43m (National Institute of Health). **B)** After 24 hours of incubation in anaerobic conditions at 37°C, the concentration of bacteria was quantified by qPCR: *S. gordonii,* black bars; *P. gingivalis,* grey bars; *T. denticola,* white bars. The data are the average of triplicate assays.

Figure 2

As demonstrated by image analysis and qPCR measurements, biofilms performed in MMBC-3 media displayed similar biomasses as biofilms in BHIe (Fig 2A), with identical ratios of each species (Fig. 2B). As expected from previous observations of

2-species biofilms (Martin *et al.*, 2017), the proportion of *S. gordonii* in three-species biofilms was always higher than those of *P. gingivalis* and *T. denticola,* probably due to its higher growth rate and antagonistic behaviour.

Conclusion

From the simplest medium composition for *P. gingivalis* (MMBC-1), supplemented media were designed and validated for the growth of *P. gingivalis* together with *T. denticola* (MMBC-2) and the growth of the two anaerobic pathogens together with *S. gordonii* (MMBC-3), allowing an increment in the complexity of the media as per need. These media are suitable for biofilm mode of growth and are specifically designed to control the levels of iron and the nature of iron sources, unlike rich media which are generally used for growing a mixed biofilm, comprising of these fastidious oral bacteria.

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Declarations of interest: none

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