

IMPROVEMENT OF AN EXPERIMENTAL MODEL OF ORAL BIOFILM

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Abstract – Objectives: The main aim of our work was to get one step closer to the *in vivo* conditions. We started from a multispecies static biofilm model containing five different bacteria, implementing specific enhancements. Our second goal was to improve the analysis of such biofilms regarding collection and identification.

Material and Methods: We started from a multispecies static model with five oral strains growing on hydroxyapatite discs to improve it on multiple points. We modified culture conditions and added two more strains. We also changed bacteria collection, which evolved from manually scrapping the discs surface to the combination of ultrasonic and mechanical harvesting. In a further another step, we developed a dynamic model implementing the above changes with a continuous supply of medium flow and waste disposal. Different methods have been evaluated to monitor the presence of all the species within the biofilms, and to quantify them: gram staining, PCR, MALDI-TOF-MS, qPCR.

Results: The modifications brought to our static model confirmed its reproducibility. Even if improvements need to be made, our dynamic model of oral biofilm is already a good alternative to more sophisticated and expensive models.

Conclusions: This new oral biofilm model represents the premises of another way to study the environmental variations effects on bacterial development, its larger application will result in a better understanding of oral health significant factors.

Keywords: Biofilm, Oral bacteria, Experimental design.

INTRODUCTION

The oral ecosystem presents a great complexity since it can harbor more than 700 different bacterial species. Most of them are organized in a biofilm on both the dental and the mucosal surfaces. Studying this complex environment is of utmost importance because a rupture in its stability can lead to the preeminence of pathogenic microorganisms, causing dental decay, gingivitis and periodontitis¹. Furthermore, various studies described the relationship between bacterial species found in the oral biofilm and their presence within other biofilms, such as heart valves biofilm².

Thus, the reproduction of oral biofilms is extremely difficult *in vitro*. This is due to the complex interrelations between all the different species and the numerous variations of their environment. Besides, growing, harvesting and counting the bacteria are three critical laboratory procedures. Even if *in vitro* models fail to re-create the complexity of the oral environment, they offer many advantages. They contribute to demonstrate the cariogenic potential of different microorganisms, or the effect of various components on oral biofilms³⁻⁶. They also help to identify the strains involved in periodontitis, and test molecules that can fight it⁷⁻⁹.

Numerous laboratory models have already been described in the literature: one of our previous works reviewed all experimental models of oral biofilms developed on inert substrates¹⁰.



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Both mono and multispecies models can be static or dynamic. Biofilm models with a single strain allow studying particular properties of the bacterium. The observation of the effect of various molecules can be done without the interference of the many interactions present in the oral cavity.

On the contrary, multispecies biofilm models are closer to *in vivo* conditions, but are very difficult to set up, and/or quite expensive.

Therefore, the main aim of this study is to get one-step closer to the *in vivo* conditions. We want to implement specific enhancements, such as adding more anaerobe strains and building a dynamic device for biofilm culture. Our second objective is to improve the analysis of such biofilms regarding collection and identification.

MATERIAL AND METHODS

Bacterial Strains and Growth Conditions

The microorganisms tested in this study were *Streptococcus mutans* ATCC 25175, *Streptococcus oralis* ATCC 20627, *Actinomyces viscosus* ATCC 15987, *Porphyromonas gingivalis* ATCC 33277, *Fusobacterium nucleatum* ATCC 10953, *Tannerella forsythia*, ATCC 43037, *Aggregatibacter actinomycetemcomitans* ATCC 33384. All strains were cryo-preserved at -80°C. Before each experiment, two subcultures were prepared in Tryptic Soy broth (Oxoid, Dardilly, France) for *Actinomyces* and *Streptococci*, Wilkins Chalgren Anaerobe broth with 300 µL of horse serum (Oxoid, Dardilly, France) for *F. nucleatum* and *P. gingivalis*, PY Medium (ATCC media, USA) with Horse Serum (5%) and N Acetyl Muramic acid (15 µg/ml) (Sigma-Aldrich, Lyon, France) for *T. forsythia*, Brain Heart Infusion (Oxoid, Dardilly, France) for *A. actinomycetemcomitans* and incubated at 37°C for 72 h.

Static Biofilm Models Assays

Basic model

According to Guggenheim's model¹¹, for four hours, each hydroxyapatite disc (Clarkson Chromatography Products Inc., Williamson, PA, USA) was placed in a well of a sterile 24-well cell culture plate and coated with 800 µL of pasteurized saliva at room temperature with gentle shaking to promote the formation of a salivary pellicle. Then, saliva was aspirated from each well and replaced with a mixture of 800 µL of new pasteurized saliva, 800 µL of fluid universal medium (FUM) and 200 µL of bacterial inoculum prepared by combining 1 ml of overnight precultures (OD_{550nm} = 1±0.02) of *S. mutans*, *S. oralis*, *A. viscosus*, *P. gingivalis* and *F. nucleatum*. After 24 h of anaerobic incubation (Genbox Anaer, BioMerieux, France), the medium was replaced with 800 µL of enriched FUM (FUMe), containing 0.15% (w/v) glucose and 0.15% (w/v) sucrose, and the plate was incubated anaerobically at 37°C, for two more days.

Modifications following Ammann et al¹²

Two strains were added to the previous model: *T. forsythia*, and *A. actinomycetemcomitans*. After 4 hours, when the salivary pellicle was formed, the medium was replaced with 750 µL of modified FUM (FUMm, enriched with 0.30% glucose), 750 µL of horse serum, 0.34 mM of NAM (N Acetylmuramic acid), 15.3 µM of hemin and 200 µL of the bacterial inoculum prepared by combining 1 ml of overnight precultures (OD_{550 nm} = 1±0.02) of all bacteria.

After 24 h of incubation in anaerobic conditions, the discs were placed in renewed medium (750 µL FUMm, 750 µL of horse serum, 0.34 mM of NAM, 15.3 µM of hemin).

Further modifications

After incubation for 4 h, instead of using FUMm, 750 µL of enriched FUM (FUMe, enriched with 0.15% glucose + 0.15% sucrose) were added. Also, 800 µL of saliva were included to the

medium, with 750 μL of horse serum, 0.34 mM of NAM, 15.3 μM of hemin, and 200 μL of the bacterial inoculum composed of a combination of 1 ml of overnight precultures ($\text{OD}_{550 \text{ nm}} = 1 \pm 0.02$) of all bacteria.

After 24 h, 750 μL of FUMe (0.15% glucose + 0.15% sucrose) were changed, 800 μL of new saliva were added with 750 μL of horse serum, 0.34 mM of NAM, 15.3 μM of hemin. 40 μL of *T. forsythia* and 40 μL of *A. actinomycetemcomitans* ($\text{OD}_{550 \text{ nm}} = 1 \pm 0.02$) were also included in the new medium.

Dynamic Biofilm Models Assays (Figure 1)

Hydroxyapatite discs were immersed in an Erlenmeyer flask containing 500 μL of FUM supplemented with hemin (15.3 μM). This Erlenmeyer flask was connected to a bottle containing 3 L of sterile FUM enriched with 0.15% sucrose and 0.15% glucose. The role of this bottle was to ensure the medium turnover during the experiment, through a peristaltic pump (Thermo Fisher Scientific, Strasbourg, France), with a flow rate of 0.6 mL/min. Waste products were evacuated into a beaker.

The device was put on a heating plate allowing the culture medium to reach a core temperature of 37°C. For inoculation, 40 ml of the mixed precultures ($\text{OD}_{550 \text{ nm}} = 1 \pm 0.02$) of all the bacteria were added.

To start the experiments, five strains have been included: *S. mutans*, *S. oralis*, *A. viscosus*, *F. nucleatum* and *P. gingivalis*. Renewal of growth media began 24 hours after inoculation, at the described flow rate. Total incubation time was 72 hours, and then, the discs were harvested as described below. In a second protocol, we added two more strains, *A. actinomycetemcomitans* and *T. forsythia*.

Harvesting the biofilms

For all the biofilm models, the discs were harvested with the same protocol.

The discs were washed twice with a saline solution to eliminate non-adherent bacteria, and placed in test tubes with 1 ml of a saline solution. The tubes were sonicated for 30 sec at 25 W and vortexed for 2 min (TopMix, Thermo Fisher Scientific, Strasbourg, France) to remove the bacteria from the disc surfaces.

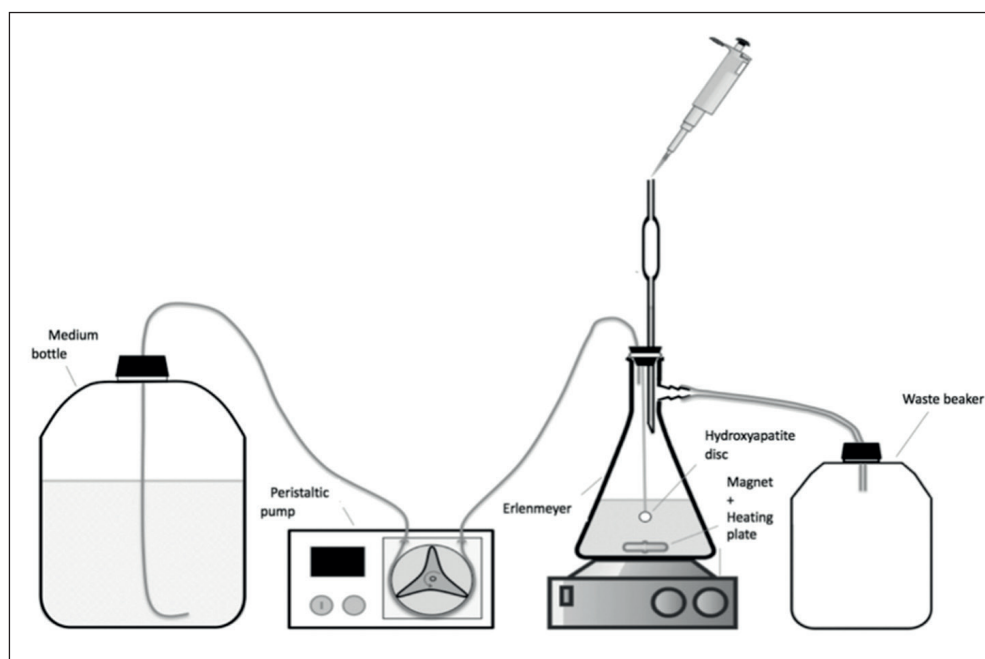


Figure 1. Schematic diagram of a dynamic multispecies biofilm device.

Concerning the static models, aliquots of harvested biofilms were diluted and spiral-plated onto Mitis Salivarius agar + tellurite (Fischer Scientific, Strasbourg, France) for *Streptococci*, Columbia blood agar (Oxoid, Dardilly, France) for *Actinomyces* and *Aggregatibacter*, Wilkins and Chalgren Anaerobe agar supplemented with blood and GN supplement (Oxoid, Dardilly, France) for *Fusobacterium*, *Tannerella* and *Porphyromonas*.

Identification

Qualitative methods (Gram staining, MALDI-TOF, PCR) have been used to ensure that each strain inoculated was found in the biofilm. A quantitative method (qPCR) was also used.

MALDI-TOF-MS

MALDI-TOF-MS was performed only for the five species dynamic biofilm, on isolated colonies. The strains were grown on Columbia agar supplemented with 5% sheep blood (Oxoid) and incubated from 48 h (for the facultative anaerobes) to 96 h (for the strict anaerobes) at 37°C.

We used the extended direct transfer technique: an isolated colony was dropped off on a target plate and allowed to dry at room temperature. 1 µL of formic acid (70%) was added to each spot and left to dry again. The dried mixture was overlaid with 1 µL of HCCA matrix. The samples were then processed in the MALDI-TOF-MS spectrometer (Ultraflex III TOF/TOF, Bruker Daltonics).

PCR (Table 1)

PCR was performed after DNA extraction (GenElute™ Bacterial Genomic DNA Kit, Sigma-Aldrich, Lyon, France).

qPCR

Genomic DNA from the harvested biofilms was extracted using GenElute™ Bacterial Genomic DNA Kit (Sigma-Aldrich, Lyon, France) according to the manufacturer's instructions. Extracted DNA corresponding to known numbers of bacterial cells was serially diluted and used to create standard curves (six different 10-fold dilutions were used).

For *A. actinomycetemcomitans*, *A. viscosus* and *T. forsythia*, the primer sequences and amplicons were chosen for their specificity according to Decat et al²⁰. The nucleotide Basic Local Alignment Search Tool and primer BLAST have been used to design them.

For *Streptococci*, *P. gingivalis* and *F. nucleatum* issued from Dalwai et al²¹, the primers were all checked for their specificities against the panel of oral species listed and were found to be specific to their intended targets. The primers are listed in Table 2. The thermocycling program settings are described in Table 3.

qPCR analysis was performed with a MyiQ™ Single-Color Real-Time PCR Detection System - Bio-Rad (Hercules, CA, USA).

All amplifications and detections were carried out in an optical 96-well reaction plate.

All data were analyzed by using iCycler iQ Software.

RESULTS

Static Biofilm Models Assays

With the basic model, as described in the literature, we were able to harvest *S. mutans*, *S. oralis*, *A. viscosus*, *P. gingivalis*, and *F. nucleatum*.

Biofilm with modified FUM (as described by Amman et al¹²) was able to recover *S. mutans*, *S. oralis*, *A. viscosus*, *P. gingivalis*, *F. nucleatum*, but *A. actinomycetemcomitans*, and *T. forsythia* were not detectable.

The latest modifications allowed us to harvest all seven bacteria strains placed in the biofilm.

TABLE 1. CONDITIONS OF PCR.

Strains	Primers	5'-3'	Amplicon length	PCR cycles	References
<i>S. mutans</i>	GTFBF	ACTACACTTTTCGGGTGGCTTGG	517 pb	30 sec at 95°C 30 sec at 59°C 01 min at 72°C 30 cycles	13
	GTFBR	CAGTATAAGCGCCAGTTTCATC			
<i>A. viscosus</i>	SIA1	CGGCGATGTCATCAGCTT	756 pb	15 sec at 96°C 30 sec at 55°C 01 min at 72°C 32 cycles	14
	SIA 2	ATGCGGTAGTTGTCCGGTG			
<i>P. gingivalis</i>	FimA-R	CTGTGTGTTTATGGCAAACCTTC	392 pb	2 min at 94°C 30 sec at 94°C 30 sec at 55°C 30 sec at 72°C 30 cycles	15
	FimA-R	AACCCCGCTCCCTGTATTCCGA			
<i>F. nucleatum</i>	FADA-F	CACAAGCTGACGCTGCTAGA	232 pb	04 min at 94°C 30 sec at 94°C 30 sec at 55,8°C 40 sec at 72°C 30 cycles	16
	FADA-R	TTACCAGCTCTTAAAGCTTG			
<i>S. oralis</i>	MKR-F	TCCCGGTCAGCAAACCTCCAGCC	374 pb	9 min at 95°C 30 sec at 94°C 1 min at 66°C 30 cycles	17
	MKR-R	GCAACCTTTGGATTTGCAA			
<i>A. actinomyces comitans</i>	AA-1	ATTGGGGTTTAGCCCTGGT	194 pb	5 min at 94°C 30 sec at 94°C 1 min at 55°C 30 sec at 72°C 30 cycles	18
	AA-2	GGCACAAACCCATCTCTGA			
<i>T. forsythia</i>	TF-1	GCGTATGTAACCTGCCCGCA	641 pb	3 min at 94°C 1 min, at 94°C 1 min, at 60°C 1 min at 72°C 35 cycles	19
	TF-2	TGCTTCAGTGTCAGTTATACCT			

Dynamic Multispecies Biofilm Device

Biofilm development

First, the culture of the bacteria on their specific medium allowed the development of colonies from each strain. Then, Gram staining and microscopic observation confirmed the identification of all strains.

The extended direct transfer method of MALDI-TOF MS confirmed the identification of only four of the five strains, *S. mutans*, *S. oralis*, *A. viscosus* and *P. gingivalis*. We were not able to identify *F. nucleatum*.

qPCR

The results for *A. viscosus* and *T. forsythia* were not exploitable. qPCR confirmed the identification and provided semi-quantitative results on *S. mutans*, *S. oralis*, *F. nucleatum*, *P. gingivalis*, *A. actinomyces comitans*.

The qPCR results demonstrated that the five standard curves, representative of typical experiments, were linear generated from a plot of C_t against log concentration for the known standard DNA for *S. mutans* ($r^2 = 0.986$, $E = 71.1\%$), for *S. oralis* ($r^2 = 0.981$, $E = 77.1\%$), for *F.*

TABLE 2. PRIMERS FOR QPCR.

Strains	Primers	5'-3'	Target genes	Amplicon length
<i>S.oralis</i> <i>S.mutans</i>	STREP-F STREP-R	GATACATAGCCGACCTGAG CCATTGCCGAAGATTCC	ARNr 16S	–
<i>A.viscosus</i>	AV-F AV-R FAM-TAMRA	ATGTGGGTCTGACCTGCTGC CAAAGTCGATCAGCTCCG ACGGAGGTCGGGAACGGTGAAG	–	96 bp
<i>P.gingivalis</i>	PG-F PG-R	GGAAGAGAAGACGTAGCACAAGGA GAGTAGGCGAAACGTCCATCAGGTC	rpoB	143 bp
<i>F.nucleatum</i>	FN-F FN-R	GGATTTATTGGGCGTAAAGC GGCATTCTACAAATATCTACGAA	–	162 bp
<i>T.forsythia</i>	TF-F TF-R	GGGTGAGTAACGCGTATGTAACCT ACCCATCCGCAACCAATAAA	ARNr 16S	127 bp
<i>A.actinomyce- temcomitans</i>	AA-F AA-R	CTTACCTACTCTTGACATCCGAA ATGCAGCACCTGTCTCAAAGC	ARNr 16S	–

nucleatum ($r^2=0.934$, $E=86.8\%$), for *P. gingivalis* ($r^2=0.984$), and *A. actinomycetemcomitans* ($r^2=0.990$, $E=86.8\%$). Four of the five melting curves (for *S. mutans* at 86°C , *S. oralis* at 86°C , *F. nucleatum* at 81.5°C , and *A. actinomycetemcomitans* at 80.5°C) demonstrated homogeneous products without primer-dimers and all the amplifications of biofilm samples were linear generated within the standard range. For *P. gingivalis*, as some biofilm samples showed heterogeneous products, those have been removed for the analysis. The linear standard curve was generated based on the log concentration of known DNA samples of the strains.

For each bacterial species, final concentrations were calculated according to the following formula:

$$X = \frac{CT_{\text{Tech}} - y_{\text{int}}}{\text{slope}}$$

The average concentrations shown in Table 4.

of each species for all experiments are

TABLE 3. THERMOCYCLING PROGRAMS FOR QPCR.

Thermocycling program for Streptococci, <i>P. gingivalis</i> , <i>F. nucleatum</i> , <i>T. forsythia</i> and <i>A. actinomycetemcomitans</i>				
Cycles	Repetition	Step	Temperature	Time
1	1 X	Step 1	95.0°C	10 min
2	40 X	Step 1	95.0°C	30 sec
		Step 2	55.0°C	1 min
		Step 3	72.0°C	1 min
Real-Time Data collection and Real-Time Analysis activated				
3	71 X	Step 1	60.0°C - 95.0°C	10 sec
4	1 X	Step 1	25.0°C	-
Thermocycling program for <i>A. viscosus</i>				
Cycles	Repetition	Step	Temperature	Time
1	1 X	Step 1	50.0°C	2 min
2	1 X	Step 1	95.0°C	10 min
3	60 X	Step 1	95.0°C	15 sec
		Step 2	58.0°C	1 min
Real-Time Data collection and Real-Time Analysis activated				
4	1 X	Step 1	25.0°C	-

TABLE 4. CONCENTRATIONS FOUND WITH QPCR.

<i>S. mutans comitans</i>	<i>S. oralis</i>	<i>P. gingivalis</i>	<i>F. nucleatum</i>	<i>A. actinomycetem</i>	
Concentration	160 CFU/μL	46 CFU/μL	6492 CFU/μL	120 CFU/μL	1.50 CFU/μL
Concentration Ratio (Standard Deviation)	$3,81.10^{-1}$ $\pm 1,78.10^{-1}$	$2,88.10^{-1}$ $\pm 1,20.10^{-1}$	$8,37.10^{-3}$ $\pm 1,38.10^{-3}$	$2,85.10^{-1}$ $\pm 1,99.10^{-1}$	$1,46.10^{-6}$ $\pm 3,5.10^{-7}$

DISCUSSION

The study of oral biofilms is very important to anticipate and control the development of oral diseases. Therefore, the aim of our work was to set up a reproducible, reliable dynamic model, which got one-step closer to the oral *in vivo* environment.

Drawing inspiration from Guggenheim's static model, we modified several conditions, following the recommendations of Amman et al¹². Then, we implemented our own further protocol modifications. We aimed to optimize those laboratory models as they lacked reproducibility, according to our preliminary studies. We shifted from classic Fluid Universal Medium to FUM enriched with 0,15% glucose and 0,15% sucrose. The addition of saliva at +4 h and +24 h simulated the oral conditions as the oral biofilm develops on surfaces coated with saliva.

We also modified the way to harvest the biofilm developed on hydroxyapatite discs. Manual scraping of the disc surface was too operator-dependent, so we chose to use the protocol described by Ready et al²² who added a sonication step after vortexing. These modifications confirmed that our static model was reproducible.

The major leap in this work has been the creation of a dynamic model from our static one. This would allow future studies to control for variations in environmental parameters, such as pH, temperature and anaerobiosis. This dynamic device renews continuously the medium and evacuates wastes like in the mouth. For now, it can support the development of seven strains together: *S. mutans*, *S. oralis*, *A. viscosus*, *P. gingivalis*, *F. nucleatum*, *A. actinomycetemcomitans*, and *T. forsythia*.

For biofilm identification, quantification by CFU numeration does not appear to be a good option. Indeed, we observed that the morphology of biofilm strains colonies was smaller than the planktonic morphology and that the different species could not be distinguished by their shape. We identified the species in our biofilms with microscopy after Gram Staining and PCR.

Although PCR is routinely used, its numerous steps (DNA extraction, PCR and agarose gel electrophoresis) are time-consuming. Therefore, in order to follow our main goal of optimizing our model, we began investigating a complementary way to recognize our strains, using Matrix-Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry (MALDI-TOF MS).

In fact, MALDI-TOF-MS is a fast (<1 hour) and efficient procedure for bacterial identification. However, so far, several scholars²³ have shown that the identification of anaerobes is the main shortcoming of this method. MALDI-TOF seems to be satisfactory for genus identification, but insufficient for identification at the species level^{24,25}. Some authors²⁶ suggested the creation of a database helping this identification. This will facilitate the step of bacterial identification in our dynamic model of oral biofilm.

qPCR confirmed the identification of five of the strains (*S. mutans*, *S. oralis*, *F. nucleatum*, *P. gingivalis*, *A. actinomycetemcomitans*) and gave quantitative information on their growth in the biofilm. This technique skips the agar culture step, and allows the detection and the qualitative and quantitative identification of the strains directly from aliquots of the harvested biofilms. Nevertheless, further experiments are needed to carry out a quantitative analysis for *A. viscosus* and *T. forsythia*. So far, qPCR has not shown positive results for these two strains, although it has followed the protocols described in the literature.

Culture-dependent (culturomic) and culture-independent methods seem to be complementary. The use of these two approaches could optimize bacterial characterization²⁷.

Our model can be compared to the constant depth film fermentor (CDFF), which is known as one of the most advanced *in vitro* biofilm models currently available. Both are sterilizable,

allow the aseptic removal of samples and can contain any material as a substrate. Our model presents some benefits compared to the constant depth film fermentor, as the question of reproducibility using the CDFE has to be discussed, according to Kinniment et al²⁸: it remains a versatile method for producing reproducible aliquots of oral biofilms²⁹. Our model showed a better ability to repetitively grow multispecies oral biofilms. Moreover, its implementation is less expensive than the CDFE.

The next steps in our studies are to add more bacterial species to develop a more complex biofilm, and to study the effects of environmental variations on biofilm development. To achieve these objectives, we plan to include another difficult-to-grow anaerobic strain: *T. denticola*, which is frequently described as involved in periodontitis, and to change environmental conditions in our biofilm model (sugars input, change of pH...), in order to study their influence on biofilm growth.

CONCLUSIONS

This new oral biofilm model represents the premises of another way to study the environmental variations effects on bacterial development. Its application will result in a better understanding of oral health significant factors.

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Conflict of Interest

The authors declare that they have no conflict of interests.

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