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1 The role of saliva in the persistence of *Streptococcus*
2 *gordonii* in water

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16

17 **Running title**

18 Saliva promotes *S. gordonii* survival

19 .

20 **1. Abstract**

21 **Introduction:** *Streptococcus gordonii* is one of the pioneer colonizer of the oral
22 cavity and may be isolated from dental unit waterlines (DUWL) mixed with saliva
23 because of antiretraction valve dysfunction.

24 **Aims:** The behavior of *S. gordonii* in tap-water and the influence of low saliva
25 concentrations (0-10% v/v) on bacterial proliferation were investigated. We
26 evaluated the effect of saliva on bacterial survival in tap-water in order to know if
27 it could facilitate or not the biofilm formation in the context of DUWL.

28 **Materials and Methods:** *S. gordonii*'s (DL1) survival ability was evaluated in
29 tap-water with or without saliva supplementation (0-10% v/v) at 20°C and 27°C.
30 Whole unstimulated saliva (WUS) and artificial saliva (AS) were both tested in
31 this study. A gas chromatography and mass spectrometry (GC/MS) analysis was
32 performed on filtered lyophilized WUS and the effect of each AS's components
33 were evaluated on *S. gordonii*'s survival.

34 **Results:** *S. gordonii* survived in tap-water no longer than : 24h at 27°C and 72h at
35 20°C ; water supplementation with saliva induced the bacterial inoculum
36 maintained at 20°C (> 1% v/v) and enabled bacteria to survive up to 7 days at
37 27°C (≥ 5% v/v). Various most simple structural artificial saliva's compounds
38 (both proteic and carbohydrate) seemed participate to this survival improvement.

39 **Discussion:** Even if saliva contains some antimicrobial agents like lysozyme, it
40 can also have a positive effect on *S. gordonii*. Very few informations are known
41 on saliva's compounds responsible of this effect.

42 **Conclusion:** Saliva, even in low proportions (1-10% v/v), was able to facilitate
43 the survival of *S. gordonii* in tap-water. Several of its components seems
44 participate on this effect.

45 **Keywords:** Oral microorganisms, biofilm, tap-water, dental care.

46 **Introduction:** *Streptococcus gordonii*, colonisateur précoce de la cavité buccale,
47 est potentiellement isolé dans les lignes d'eaux des units de soins dentaires en
48 même temps que de la salive suite à un dysfonctionnement des valves anti-reflux.

49 **Buts:** Le comportement de *S. gordonii* dans l'eau potable et l'influence de faibles
50 concentrations en salive (0-10% v/v) sur la prolifération bactérienne ont été
51 investigués. Nous avons évalué l'effet de la salive sur la survie bactérienne dans
52 l'eau dans le but de savoir si la salive pouvait faciliter ou non la formation de
53 biofilm dans le contexte des lignes d'eau des units de soins dentaires.

54 **Matériels et méthodes :** La capacité de survie de la souche DL1 de *S. gordonii* a
55 été évaluée dans de l'eau potable, additionnée ou non de salive (0-10% v/v), à
56 20°C et 27°C. De la salive entière non stimulée (fluide buccal humain) et de la
57 salive artificielle ont été testées dans cette étude. Une analyse par
58 chromatographie en phase gazeuse couplée à une spectrométrie de masse a été
59 réalisée sur un filtrat lyophilisé de salive entière non stimulée. L'influence de
60 chacun des constituants de la salive artificielle sur la survie de *S. gordonii* a été
61 étudiée.

62 **Résultats :** *S. gordonii* ne survit pas plus de 24h à 27°C et 72h à 20°C dans l'eau ;
63 l'ajout de salive (> 1% v/v) dans l'eau entraîne le maintien de l'inoculum

64 bactérien initial à 20°C et permet la survie bactérienne jusqu'à 7 jours à 27°C (\geq
65 5% v/v). . Plusieurs des plus simples composés structuraux de la salive artificielle
66 contribueraient à augmenter la survie bactérienne, incluant des composés
67 protéiques et glucidiques.

68 **Discussion :** Même si la salive contient certains agents antimicrobiens tels que le
69 lysozyme, elle peut aussi avoir un effet favorable sur *S. gordonii*. Très peu
70 d'informations sont connues sur les composants de la salive responsables de cet
71 effet.

72 **Conclusion :** La salive, même en faibles proportions (1 à 10% v/v) est capable de
73 faciliter la survie de *S. gordonii* dans l'eau. Plusieurs de ses composants semblent
74 participer à cet effet.

75 **Mots-clés :** Microorganismes buccaux, biofilm, eau potable, soins dentaires.

76 **2. Introduction**

77 The major source of the microorganisms isolated in the output water of dental unit
78 waterlines (DUWL) is incoming main water. However, the possible absence or
79 probable dysfunction of anti-retraction valves results in aspiration of up to 22 μ L
80 of oral fluid each time the turbine is stopped (Bagga *et al.*, 1984, Montebugnoli &
81 Dolci, 2000, Pankhurst, 2003) ; this can lead to contamination of DUWL with oral
82 microorganisms. Indeed, bacteria of the genus *Streptococcus* are found in up to
83 85% of output water samples from handpieces (Walker *et al.*, 2000, Szymańska *et*
84 *al.*, 2008). The study focused on *Streptococcus gordonii* which is a commensal
85 organism of the oral cavity and a pioneer colonizer of oral cavity surfaces (Kreth

86 *et al.*, 2008, Nobbs *et al.*, 2010). In addition, it promotes complex biofilm
87 formation: some microbial species normally poorly able to form monospecies
88 biofilms have been shown to form mixed biofilms with *S. gordonii*. As a result,
89 oral microorganisms including *S. gordonii* may colonize DUWL, grow into a
90 complex biofilm mixed with environmental microorganisms and disseminate in
91 the water following detachment of sessile cells. However, *S. gordonii* cells from
92 the oral cavity face a very unfavorable environment in DUWL: lowered and
93 unsuitable temperature, modified pH, lack of nutrients, etc. The aim of this study
94 was to investigate the ability of *S. gordonii* to survive in tap-water and the
95 potential influence of the saliva that may have simultaneously penetrated in
96 DUWL, in low proportions, due once again to the dysfunction of anti-retraction
97 valves. This degree of survival ability may enable *S. gordonii* to fit into the multi-
98 species biofilm already developed on pipe surfaces and thereby encourage
99 adhesion of other potentially pathogenic species to the oral cavity.

100 **3. Materials and Methods**

101 The survival ability of *S. gordonii* (DL1) was investigated in tap-water
102 supplemented with different concentrations of saliva; whole unstimulated saliva
103 (WUS) and artificial saliva (AS) were both tested. Experiments were performed at
104 2 temperatures (20°C and 27°C) compatible with those found in DUWL.

105 WUS was collected on ice from 11 healthy adult volunteers who gently rinsed
106 their mouth with sterile water before sampling, in order to decrease bacterial
107 contamination (Barbot *et al.*, 2011). Donors did not eat or smoke during the 2
108 hours before saliva collection and were not under antibiotherapy or

109 corticotherapy. Saliva was pooled and centrifuged (4000 g, 15 min) and then
110 filtered through a 0.22 µm membrane (Millipore) and stored at – 80°C until use.

111 AS was prepared as described by *Sissons et al.* and contained for 1L : proteose
112 peptone (10g), tryptone (5g), yeast extract (5g), KCl (2.5g), sucrose (2g), arginine
113 (0.21g), urea (60 mg), hemin (50 mg), menadione (10 mg) and hog gastric mucin
114 (2.5g) (*Sissons et al.*, 1991).

115 Regarding bacterial survival assay, *S. gordonii* strain DL1 was first cultured on
116 Columbia 3 sheep blood agar at 37°C for 48 h ; a suspension at 10⁸ cells mL⁻¹ was
117 then incubated at 20°C or 27°C for 168 h, in 96-well microplates (Costar®,
118 Corning, New-York, USA), in tap-water filtered at 0,22 µm and supplemented
119 with WUS (either centrifugated and filtered or only centrifuged) or AS at
120 concentrations ranging between 1% and 10% (v/v). *S. gordonii* viability was
121 evaluated during the time course of the experiment (up to 168 h): each bacterial
122 suspension was diluted (1/10⁴; 1/10⁵ and 1/10⁶) in fresh tap-water and 100 µL
123 were then plated in duplicate on Columbia 3 sheep blood agar. Bacterial CFU
124 were enumerated after 24 h at 37°C. Each experiment was carried out twice.

125 Statistical analysis of data was performed using the StatView software.

126 Differences between groups were analyzed using the Kruskal-Wallis and Mann
127 and Whitney tests.

128 A gas chromatography and mass spectrometry (GC/MS) analysis was performed
129 on filtered lyophilized WUS. After lyophilisation, the saliva sample was
130 volatilized by pyrolysis (pyrolyzer Pyroprobe 2000, Chemical Data Systems,

131 Oxford), separated on silica capillary column (DBWAX 30 m, 0,25 mm, J&W
132 Scientific) on HP G1800A chromatograph. Fragments were analyzed by mass
133 spectrometry and the GC/MS database software allowed for identification of the
134 main components. To better characterize the mode of action of saliva, the
135 influence of each component of AS, taken separately in tap-water at its original
136 concentration, was evaluated on the bacterial concentration until 72h at 27°C.

137 **4. Results**

138 Results showed that at 20°C in tap water without saliva, *S. gordonii* concentration
139 was maintained up to 48 h at about 10^7 CFU.mL⁻¹ and decreased at about 10^5
140 CFU.mL⁻¹ after 72 h; Figure 1A (Fig. 1A). On the contrary, at this temperature, *S.*
141 *gordonii* cells incubated in tap-water supplemented with WUS maintained a stable
142 concentration at about 10^7 CFU.mL⁻¹ throughout the study, regardless of the
143 saliva concentrations tested; Figure 1A (Fig. 1A). *S. gordonii* concentration was
144 significantly higher ($p \leq 0.005$) in water supplemented with saliva (1 to 10% (v/v))
145 after 72h. Similar results were obtained using centrifuged WUS or both
146 centrifugated and filtered WUS (data not shown). *S. gordonii* cells did not survive
147 more than 24 h at 27°C in tap-water without saliva, thereby suggesting bacterial
148 sensitivity higher at 27°C than at 20°C. A time- and dose-dependent effect of
149 saliva was observed at 27°C since *S. gordonii* cells were able to survive up to 168
150 h only in the water supplemented with the highest saliva concentrations: 5% and
151 10% v/v; despite the use of such high saliva concentrations, a decrease of about
152 10^3 or 10^4 CFU was observed throughout the study ($p \leq 0.0003$); Figure 1B (Fig.
153 1B). Tap-water supplemented with 1% or 2% saliva enabled *S. gordonii* to survive

154 up to 48h at 27° C; that said, decreased bacteria concentration of about 10² CFU
155 was observed ($p \leq 0.0003$). Similar results were obtained using water supplemented
156 with AS instead of WUS (data not shown). Furthermore, a sonication step was
157 added prior to the suspension plating so as to determine whether or not the
158 decreased bacterial concentration observed in water without saliva was due to
159 increased mortality related to severe conditions. Another hypothesis could be that
160 the adhesion of bacteria to well walls forms a biofilm. No difference in bacterial
161 concentrations was observed before or after sonication, thereby demonstrating that
162 supplementation of tap-water with saliva (WUS or AS) promoted *S. gordonii*
163 survival. Concerning the influence of AS components, results showed that
164 proteose peptone, tryptone, yeast extract, KCl, sucrose and arginine taken
165 separately significantly improved ($p < 0.01$) *S. gordonii*'s survival in tap-water, at
166 27°C until 72h of incubation; Figure 2 (Fig 2). On the contrary supplementation
167 with urea, hemin, menadione and hog gastric mucin taken separately did not
168 significantly increase bacterial survival.

169 5. Discussion

170 As expected, tap-water was not an appropriate medium for *S. gordonii*, which
171 required a richer environment to optimize its growth. As previously shown in
172 other oral microorganisms, these results suggest that addition of saliva to the
173 water could provide *S. gordonii* with some essential components (Barbot *et al.*,
174 2011). However, unlike previously observed studies of the influence of saliva on
175 *Candida* yeasts, saliva was unable to induce the proliferation of *S. gordonii* in tap-
176 water, regardless of the tested concentration (Barbot *et al.*, 2011); saliva activity

177 would consequently only slow *S. gordonii* cell death in tap-water, potentially
178 giving the cells enough time to find a protected environment, such as the biofilm
179 that normally preexists in DUWL. In addition, temperature could play an
180 important role in bacterial maintenance, since survival of *S. gordonii* was longer
181 at 20°C than at 27°C. The average temperature of most DUWLs is
182 23°C(Pankhurst, 2003).

183 The effect of saliva on microorganisms has been investigated primarily in
184 experimental conditions mimicking the mouth environment; for example, it has
185 been shown that whole saliva would promote the adherence of *C. albicans* to
186 silicon in a dose-dependent manner (Holmes *et al.*, 2006). In addition, O'Sullivan
187 *et al.*(7) showed that *C. albicans* adhesion to oral streptococci was promoted by
188 the selective adsorption of salivary proteins (salivary basic proline-rich proteins)
189 to the streptococcal cell surface, thereby suggesting an interaction between oral
190 streptococci and the proteic components of saliva (O'Sullivan *et al.*, 1997). This
191 finding would be in agreement with the hypothesis of a potentially key role of
192 saliva proteins in interactions with microorganisms.

193 GC/MS analysis of saliva samples demonstrated the mainly proteic molecular
194 composition of saliva, including components such as indole and toluene;
195 furthermore some organic acids and aromatic molecules were also present and
196 only one carbohydrate (acetamide) was found; Figure 3 (Fig. 3).Results obtained
197 while studying the separate and specific influence of each saliva components,
198 suggested that *S. gordonii* could use proteic components as well as carbohydrates
199 to survive, provided that their structure be readily degradable.

200 To conclude, these results highlighted the positive influence of saliva on the
201 survival of *S. gordonii* in a drastic environment such as tap-water, even with a
202 very low concentration of saliva (about 1%); the favorable influence was obvious
203 at both 20°C and 27°C which correspond to the temperatures that frame the
204 thermal variations of DUWL. *S. gordonii* cells contaminating DUWL may thereby
205 survive and join the complex biofilm already formed on DUWL surfaces; their
206 attachment will certainly be facilitated by the adherent properties of *S. gordonii*,
207 which is well-known as a pioneer colonizer. In addition, preliminary results
208 suggested that *S. gordonii* could secrete molecules likely to improve the growth of
209 other oral bacteria in tap-water (data not shown). Successful maintaining of *S.*
210 *gordonii* in water could help oral pathogenic human species to persist in DUWL.

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216 read and reviewed the original English-language text.

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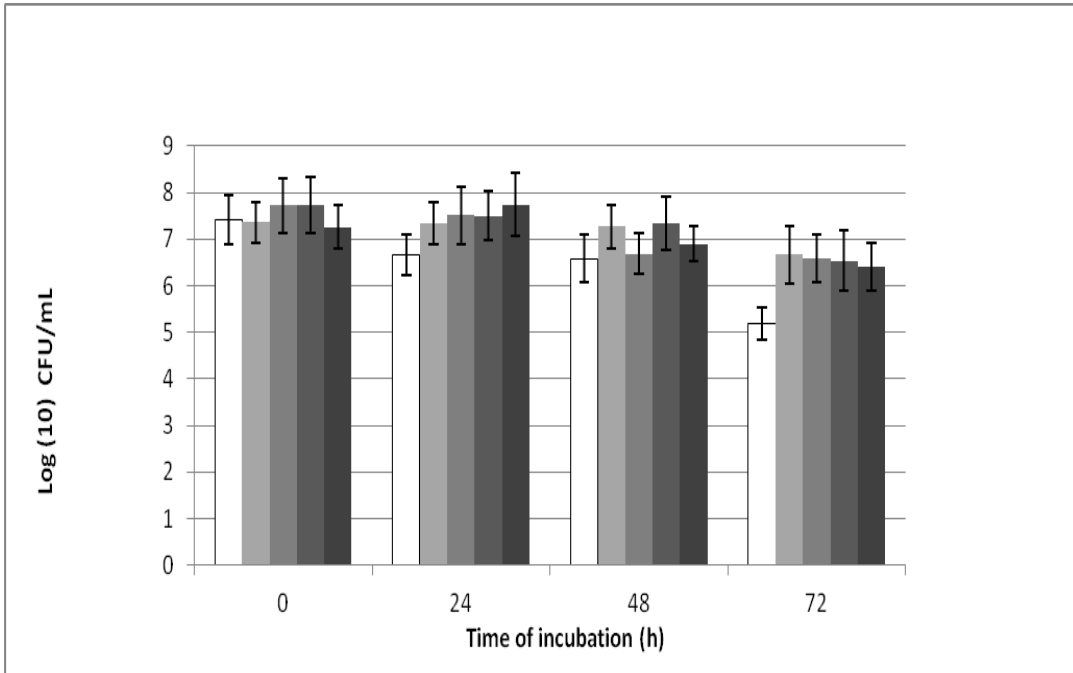
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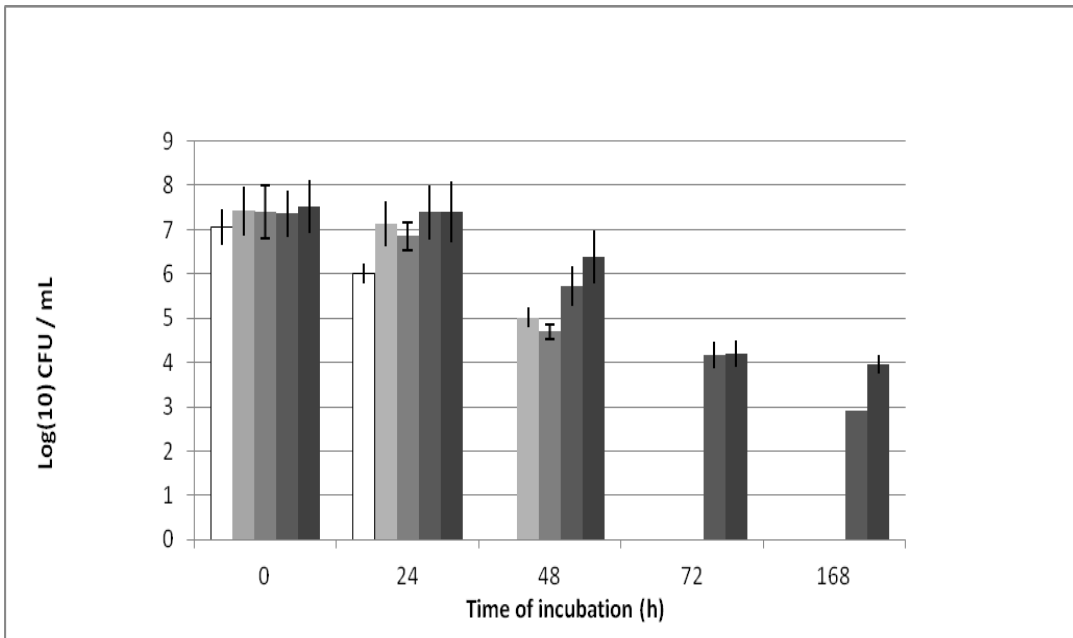
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263 **8. Figures**

264 **Fig. 1a**



265 **Fig. 1b**



266

267 **Fig. 1. Influence of saliva and temperature on *S. gordonii* survival in tap-**
268 **water**

269 Streptococcal survival was studied with different concentrations of saliva (1%,
270 2%, 5% and 10% v/v) and expressed as log(10) CFU/mL tap water. The control
271 corresponded to *S. gordonii* incubated in tap water without saliva. (A) survival of
272 *S. gordonii* at 20°C (B) survival of *S. gordonii* at 27°C. All data shown were
273 executed with saliva both centrifugated and filtered but results with saliva only
274 centrifuged were similar (data not shown).

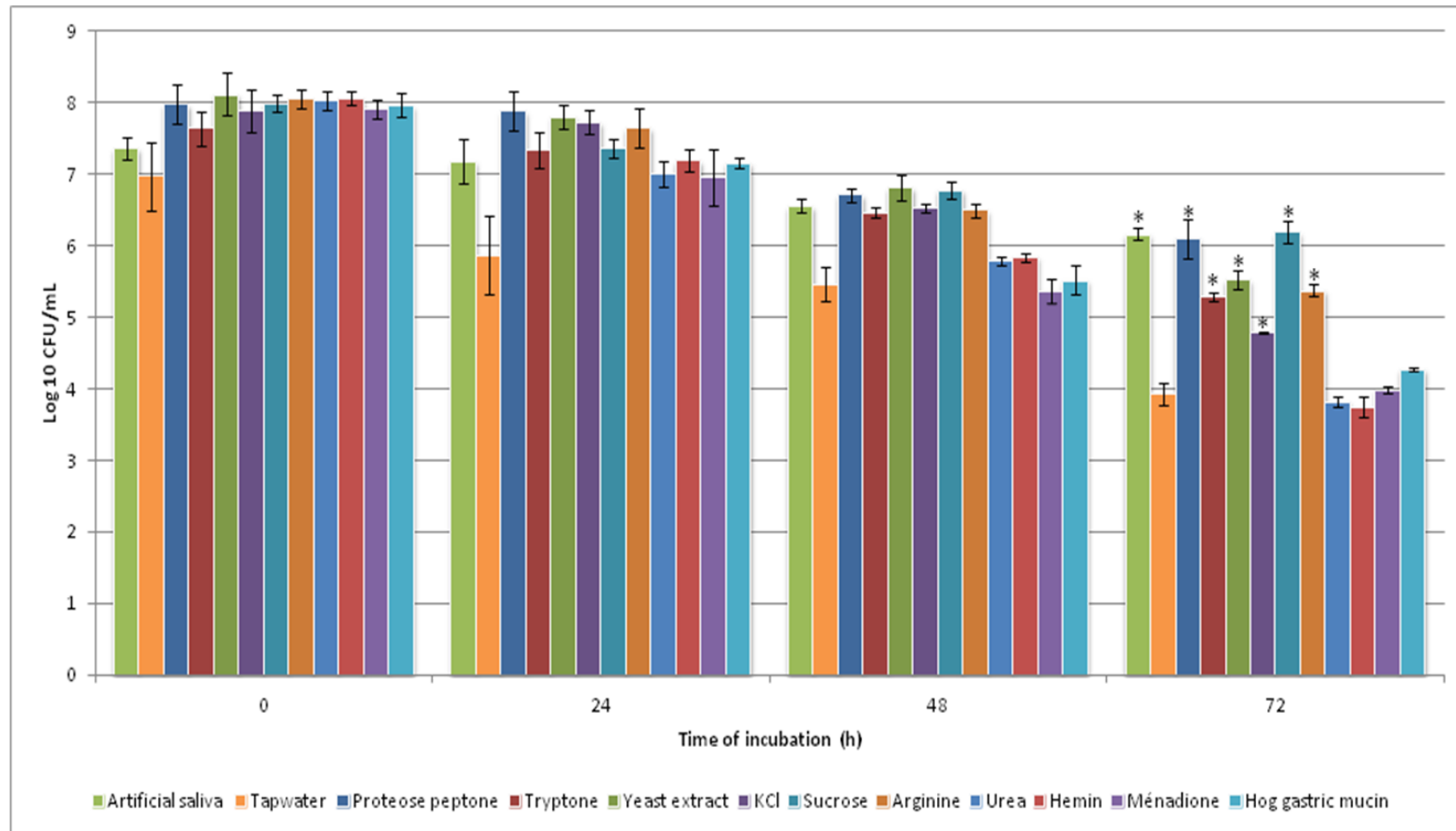
275 **Fig. 1. Influence de la salive et de la température sur la survie de *S. gordonii***
276 **dans l'eau**

277 La survie de *S. gordonii* est étudiée avec différentes concentrations de salive (1%,
278 2%, 5% et 10% v/v) dans l'eau et est exprimée en log(10) d'UFC/mL. Le contrôle
279 correspond à *S. gordonii* incubé dans l'eau sans salive. (A) Survie de *S. gordonii* à
280 20°C. (B) Survie de *S. gordonii* à 27°C. Toutes les données représentées ici ont
281 été réalisées avec de la salive à la fois centrifugée et filtrée mais les résultats
282 obtenus en salive uniquement centrifugée été similaires (valeurs non
283 représentées).

284

285

286 **Figure 2**



287

288 **Fig. 2. Impact of Artificial Saliva's (AS) components on *S. gordonii*'s survival in water**

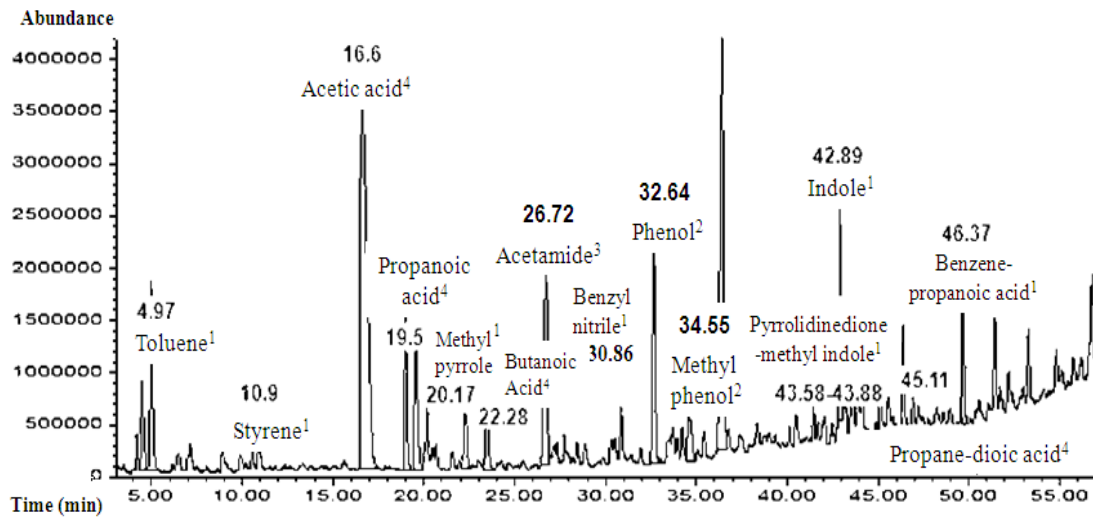
289 Each of the AS's component was evaluated on his effect on the *S. gordonii*'s survival in water at 27°C until 72h. * p<0.01

290 **Fig. 2. Impact des composants de la salive artificielle sur la survie de *S. gordonii* dans l'eau**

291 Chacun des composants de la salive artificielle a été évalué sur son effet sur la survie de *S. gordonii* dans l'eau à 27°C jusqu'à 72h. *

292 p<0.01

293 **Figure 3**



294

295 **Fig. 3. Chromatogram of filtered saliva, analysed by GC/MS.**

296 ¹proteins ; ²aromatic molecules ; ³carbohydrates ; ⁴organic acids

297 **Fig. 3. Chromatogramme de la salive filtrée, analysée en GC/MS.**

298 ¹protéines ; ²molécules aromatiques ; ³sucres ; ⁴acides organiques

299