

1 **Evaluation of the risk of fungal spoilage when substituting sucrose with**
2 **commercial purified Stevia glycosides in sweetened bakery products**

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31 **Keywords:** Stevia, sweeteners, water activity, *Eurotium*, *Aspergillus*, *Penicillium*,
32 bakery products

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35

36 **Abstract**

37

38 The objectives of this study were to compare the addition of different Stevia-based
39 sugar substitutes (S1-S3) with sucrose alone and sucrose + S1 on (a) humectant a_w
40 properties, (b) relative colonisation rates of sponge cake slices at 0.90 a_w by strains of
41 *Aspergillus flavus*, *Eurotium amstelodami*, *Fusarium graminearum* and *Penicillium*
42 *verrucosum* at 20 and 25°C and (c) shelf-life periods in days prior to visible growth.
43 This showed that sucrose, S1 and S1+sucrose in water solutions were able to reach a_w
44 levels similar to those of glycerol and glucose mixtures. The S2 and S3 commercial
45 sugar substitutes were unable to reduce a_w levels significantly. Colonisation of sponge
46 cake slices by *E. amsteladami*, *A. flavus* and *P. verrucosum* occurred with all the
47 treatments at 25°C. At 20°C, sucrose alone inhibited growth of *A. flavus*. *F.*
48 *graminearum* growth only occurred in sponge cake slices containing S2 and S3 Stevia-
49 based products at both temperatures. The longest shelf-life periods (30 days), without
50 spoilage, was for *A. flavus* at 20°C with sucrose and S1 Stevia product and for *E.*
51 *amstelodami* at the same temperature with sucrose. *F. graminearum* was completely
52 inhibited, with no growth occurring at both temperatures and sucrose alone, S1 and
53 sucrose + S1 treatments. This study suggests that, as part of a hurdle technology
54 approach, replacing sucrose with low calorie sugar substitutes based on Stevia
55 glycosides needs to be done with care as different products may have variable
56 humectant properties and bulking agents which may shorten the shelf-life of
57 intermediate moisture bakery products.

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59

60 Introduction

61

62 Sucrose is currently one of the main ingredients in the food industry and is especially
63 important in sweetened bakery products and confectionary for its preservation
64 characteristics and as an important source of energy, providing 394 kcal/100 g of
65 refined sugar. Its preservation properties are to act as a humectant by reducing the
66 water activity (a_w) of bakery products as part of a hurdle technology strategy.
67 Intermediate bakery products have a relatively short shelf-life although this can be
68 extended by addition of aliphatic acids and sometimes modified atmosphere
69 packaging. However, these intermediate moisture products are prone to colonisation
70 by xerophilic and xerotolerant fungi when not stored properly. The most important
71 spoilage moulds in bakery products are species from *Eurotium*, *Aspergillus* and
72 *Penicillium* (Abellana et al., 1997; Arroyo et al., 2005; Guynot et al., 2005; Spicher,
73 1980; Williams, 1990; 1999; 2001;).

74

75 There has been interest in the substitution of sucrose with alternative lower calorie
76 sweeteners in bakery products. The most common high-intensity sweeteners in the
77 world market (e.g. saccharin, aspartame, sucralose) are made of synthetic compounds
78 (Abdalbasit et al., 2014). There has thus been interest in other alternative plant-based
79 products based on extracts from the plant *Stevia rebaudiana* (Bertoni). The
80 compounds of interest are known as steviol glycosides (Boileau et al., 2012). Stevia
81 products have redefined the category of intense sweeteners globally, because, for the
82 first time, food manufacturers have access to an effective non-calorific sweetener that
83 has a 'natural' image (Gibson-Moore, 2013). In 2011, the European Commission
84 authorised the use of pure steviol glycosides (95%) in foods and beverages in the
85 European Union.

86

87 Stevia products are being increasingly used (Chattopadhyay et al., 2014) by food
88 companies which produce sweetened bakery products and recommended as a
89 substitute for home baking. However, there have surprisingly been no studies on
90 whether substituting sucrose with Stevia glycosides completely, or in combination

91 with sucrose, will result in the required target a_w levels of products such as cakes and
92 provide similar shelf-life properties as sucrose in terms of preventing fungal spoilage
93 from being initiated.

94

95 The objectives of this study were to (a) evaluate the water binding capabilities of
96 different commercial Stevia-based sweetener formulations by comparing them with
97 sucrose, glucose and glycerol, (b) to examine the effect of three Stevia substitute
98 products (S1-S3) alone or with sucrose on fungal colonisation rates on sponge cake
99 slices at 20 and 25°C by different spoilage fungi (*Aspergillus flavus*, *Penicillium*
100 *verrucosum*, *Eurotium amstelodami* and *Fusarium graminearum*), and (c) relative
101 shelf-life of different formulated sponge cake slices in terms of time (days) before
102 visible spoilage was initiated.

103

104 **2. Materials and methods**

105

106 2.1. Commercially purified Stevia products

107 Three different commercially available products were bought from UK retail shops and
108 identified as S1, S2 and S3. The information on the product labels with regard to
109 weight, composition, manufacturer dosage guidelines and energy per 100 g was
110 recorded and are shown in Table 1.

111

112 2.2. Determination of the water activity (a_w) of solutions of different sugars and 113 additives

114 RQ-Water solutions containing 5, 10, 20, 30, 40, 80 and 100 g/100ml of water were
115 prepared using the different Stevia products. Because at the higher concentrations,
116 Stevia products in water solutions were close to their maximum solubility, the
117 temperature was increased when required to allow complete dissolution.

118

119 For comparison, glycerol-water, sucrose-water and glucose-water solutions were
120 prepared, according to the literature in the range 0.995 and 0.80 water activity (a_w ;
121 Dallyn and Fox, 1980; Scott, 1957). The a_w of all solutions were then measured using

122 an Aqualab 3TE instrument (Decagon, Pullman, WA, USA). All measurements were
123 made with three replicates per treatment solution.

124

125 2.3. Preparation of the cake analogue

126

127 A sponge cake analogue similar to that used by Abellana et al. (1999) was prepared
128 for these studies. The recipe consisted of the following basic ingredients: 275 g self-
129 rising wheat flour, 250 ml of vegetable oil and 4 medium sized eggs. In this study 5
130 different treatments were included. These were (i) sucrose-based cakes (control), (ii)
131 100% substitution of sucrose by the manufacturers recommended amounts of Stevia
132 products for S1, S2 and S3 and (iii) 75 % substitution of sucrose by the Stevia product
133 S1 according to an online cake recipe.

134

135 The ingredients were mixed in a multifunctional kitchen mixer to make the cake
136 dough. The dough batches were placed in grease (vegetable oil) disposable aluminium
137 tins and baked in an oven at 160-170°C for 40-45 min.

138

139 After baking, the tins were covered with sterilised cooking foil and transferred to a
140 laminar flow bench for further processing. The cooled cakes were cut into ≈4 mm thick
141 slices and placed in sterile 9 cm Petri plates. The cake slices were then exposed to
142 254nm UV light for 10 min in a Herolab CleneCab Plus (Herolab GmbH Laborgeräte,
143 Germany) to eliminate any surface contamination. The a_w of the cake slices were
144 checked using an Aqualab 3TE instrument (Decagon).

145

146 Subsequently, all the treatments were equilibrated at 0.90 a_w in order to carry out
147 fungal growth experiments. The cake slices in Petri plates were divided into 4 groups
148 and placed in plastic chambers together with two 500ml beakers containing a glycerol-
149 water solution with an equilibrium relative humidity value identical to the a_w
150 treatments (=0.90 a_w). Equilibration was achieved by incubating for 48 h. Appropriate
151 equilibration was confirmed using an Aqualab 3TE instrument and found to be within
152 ± 0.02 of the target a_w level.

153

154 2.4. Strains used in these studies and inoculation method

155

156 Fungal strains *Aspergillus flavus* (NRRL3357), *Penicillium verrucosum* (OTA11),
157 *Eurotium amstelodami* (IMI229971) and *Fusarium graminearum* (FgB (L1-2/2D)) were
158 grown on Malt Extract Agar (MEA) for 10 days, except for the *E. amstelodami* strain
159 which was grown on MEA modified to 0.95 a_w . From these cultures spores were
160 collected using 10 mL sterile saline solution containing 0.05% Tween 80 (Acros
161 Organics, USA) and rubbing the surface with a sterile glass rod in order to remove
162 conidia. The spore suspensions were counted using a haemocytometer (Fisher
163 Scientific, United Kingdom) and adjusted to 1×10^6 spores/ml and directly used to
164 inoculate the cake slices. Each cake slice was centrally inoculated with 3 μ l of the spore
165 suspension.

166

167 2.5. Incubation and measurement of growth and data analyses

168

169 Experiments were conducted at 20 and 25°C. In all cases, observations were carried
170 out every two days or as necessary, and the diameter of the growing colonies
171 measured in two directions at right angles to each other. Growth was observed with
172 the aid of a binocular magnifier (Olympus SZ, Olympus, Japan). Measurements were
173 taken for a maximum of 1 month. All experiments were carried out with at least three
174 replicates per treatment.

175

176 The temporal colony diameters were measured and subjected to primary modelling
177 using the linear model. The maximum growth rate (μ_{max})(mm diameter/day) of each
178 fungal treatment on the different cake analogues was determined. Regression lines
179 were made using the time points which represented the linear phase of the growth
180 curves using Microsoft®Excel®:MAC 2011 (14.4.8) (Microsoft Corporation, Redmond,
181 USA). The slope of the linear equation with an associated correlation coefficient of not
182 $< R^2 = 0.98$ was considered the μ_{max} . Lag times (λ) were calculated by equalling the
183 regression lines to the size of the inoculum point.

184

185 2.6. Statistical analysis

186 The normality of the data sets was investigated using the Shapiro–Wilk test ($\alpha=0.05$).
187 This was followed by Levene's test ($\alpha=0.05$) to determine variance homogeneity. Due
188 to non-normality of the growth data, analysis was performed using non-parametric
189 tests for testing whether distributions across factor levels were centered at the same
190 location. Differences between independent groups at each temperature, using the
191 different stevia formulations, sucrose only and the sucrose-S1 mixture as factors, were
192 examined by the Kruskal–Wallis analysis of ranks. Nonparametric multiple comparison
193 using the Wilcoxon each pair test were performed to identify differences within
194 treatments. When growth occurred only under two conditions, homogeneity of
195 variance was tested and the appropriate t-tests were used. When analysing the shelf-
196 life prior to visible growth datasets values of 30 days were removed to avoid bias.
197 The statistical package JMP®12.1 Pro (SAS Institute Inc., 2015, Cary NC, USA) was used
198 in the analysis.

199

200 **3. Results**

201 3.1. Comparison of the water activity of different solutions of sugars and humectants

202

203 The three Stevia products (S1-S3) were all able to reduce the a_w of the solutions,
204 although the actual final levels differed significantly (Figure 1). The S1 Stevia product
205 was much more efficient in reducing the a_w in water solutions when compared with
206 S2 and S3. These two products showed a very similar behaviour pattern with regard
207 to modifying a_w .

208

209 Figure 1 also shows that the solubility of S1 in water was higher than for the other
210 Stevia treatments (S2 and S3). For this reason, there are more data points. For S1 the
211 solubility limit was very close to 100g/100mL of water. We observed that under these
212 conditions solubility was temperature-dependent and we had immediate
213 precipitation when the solution was cooled. However, product S1 was able to reduce
214 the a_w from 1.00 to 0.871 whilst products S2 and S3 were able to only slightly reduce
215 a_w to 0.986 and 0.987 a_w , respectively.

216

217 In comparison to other common compounds used as ingredients in bakery products
218 (sucrose, glucose and glycerol) the S2 and S3 commercial Stevia products were
219 relatively ineffective in decreasing a_w . However, S1 was able to do so. In addition, on
220 a weight/volume basis reductions were higher than those obtained with sucrose and
221 glucose. Among all compounds tested, glycerol was the most effective humectant
222 closely followed by S1 (see Figure 1).

223

224 3.2. Comparison between the a_w achieved in the sponge cakes modified with different
225 sugars

226

227 The cakes were cooled down for 120 min and then cut into slices. The a_w of the cakes
228 ranged from 0.852 (S1+Sucrose) to 0.971 a_w (S3 alone). This showed that cakes
229 prepared with only sucrose (0.869 a_w), product S1 (0.893 a_w) or their mixture
230 (Sucrose+S1) exhibited lower a_w values when compared with cake analogues baked
231 using S2 (0.949 a_w) or S3 (0.971 a_w).

232

233 3.3. Effect of treatments on colonisation by spoilage fungi

234

235 Figure 2 shows the effect of treatments at 20 and 25°C on the colonisation rates by
236 the different spoilage fungi. This shows that *E. amstelodami* was particularly tolerant
237 of all the treatments used with colonisation rates faster than for the other spoilage
238 fungi, especially *A. flavus* and *P. verrucosum* at 25°C ($p=0.0238$ and $p<0.001$
239 respectively). Sucrose alone or sucrose + S1 was also effective at reducing relative
240 growth rates of this xerophilic species. *A. flavus* was also able to grow effectively,
241 regardless of treatment at 25°C ($p=0.062$). However, at 20°C it was inhibited by
242 sucrose alone, S1 and sucrose + S1 ($p<0.0001$). For *P. verrucosum*, while growth was
243 relatively slower, colonisation was observed for all the treatments at both 20 and
244 25°C. For both temperatures significant differences were observed between
245 different treatments (20°C $p=0.0004$, 25°C $p<0.0001$) where they grew faster with
246 products S2 and S3. *F. graminearum* was the most sensitive species tested and it was
247 only able to grow in the presence of S2 and S3. Between them, growth with S2 was

248 significantly higher ($p=0.0043$). It was inhibited in the other treatments at both
249 temperatures.

250

251 3.4 Effect of sugar/Stevia treatments on shelf-life prior to visible growth

252

253 Table 2 shows the shelf-life in terms of number of days prior to any visible growth
254 being observed. For both *A. flavus* and *F. graminearum* at both 20 and 25°C the
255 sucrose alone and S1 treatments inhibit growth for the maximum observation period
256 of 30 days. For the latter species, a mixture of sucrose + S1 also controlled growth for
257 30 days. However, the shelf-life in the S2 and S3 products were significantly shorter
258 (at 20°C all p -values for comparisons between S2 and S3 and the other treatments
259 were <0.0275).

260

261 Sucrose was able to completely inhibit growth of *E. amstelodami* at 20°C. For the other
262 treatments, at both temperatures, the shelf-life was <4 days. For *P. verrucosum* shelf-
263 life was very short for all treatments at both temperatures, being <5 days, except for
264 sucrose at 20°C where this was 7 days.

265

266 4. Discussion

267

268 This study suggests that while Stevia glycosides may have many positive
269 characteristics as a low calorie sugar substitute, it may not be as effective as part of a
270 hurdle technology approach to control fungal spoilage unless used with other hurdles
271 in intermediate moisture bakery products. It is interesting to note that the a_w
272 reduction of different concentrations of Stevia products was variable. This may partly
273 be because of the formulation of the products themselves and their solubility. Thus
274 while S1 was effective at reducing the a_w in mixed water solutions down to levels
275 achieved with sucrose, glucose and glycerol. However, the S2 and S3 commercial
276 products did not reduce the a_w significantly.

277

278 S1 has only 1% of steviol glycosides, with the bulking agent being erythritol. Erythritol
279 is a 4-C sugar alcohol which acts as a compatible solute in fungi and is a very effective

280 humectant, almost as good as glycerol. It also occurs naturally in a number of
281 horticultural products, is heat stable up to 160°C, non-caloric and non-glycemic.
282 Studies of a_w tolerance by mycotoxigenic spoilage fungi have shown high levels of
283 biosynthesis of endogenous erythritol as a mechanism of adaptation to low a_w
284 environments (Nesci et al, 2004; Ramirez et al, 2004). Conversely, in Stevia products
285 S2 and S3, 2% of steviol glycosides were bulked with maltodextrin. Maltodextrins are
286 starch-derived α (1→4)-linked glucose polymers up to 7 to 8 glucose units. They are
287 common food additives used as a thickening or filling agent in a range of commercial
288 foods and beverages. It is nearly tasteless but is often described as being slightly
289 sweet. However, the density (g/volume) of both maltodextrin-bulked Stevia-based
290 products was very low, and if the manufacturer recommendations are followed (1
291 teaspoon is equivalent to 1 teaspoon of sucrose) only 1/10 of the weight is consumed.
292 This, coupled with the inability of maltodextrins to capture water are the reasons why
293 very little reduction of a_w was observed when substituting sucrose with products S2
294 and S3 in the sponge cake studies. Overall, the lowest a_w was obtained in the cake
295 where the mixture S1+Sucrose was used (0.852 a_w). This was just slightly lower than
296 when comparing with the sucrose-baked sponge cake.

297

298 The effect of different sponge cake treatments in terms of fungal colonisation and
299 shelf-life were subsequently standardised to maintain an
300 a_w of 0.90 to eliminate any differences with regard to the original a_w levels. This
301 allowed us to make comparisons of colonisation rates by the four different fungi. It
302 was clear that the use of Stevia products S2 and S3 supported the colonisation of the
303 sponge cake slices by all the species at both 20 and 25°C.

304

305 With these products the spoilage fungi also had very short lag times prior to visible
306 growth suggesting rapid potential for contamination after baking of the product if
307 contaminated with spores of these species. The ability of maltodextrin to enhance
308 fungal growth on plants and potentially increase the amount of mycotoxins has been
309 raised by EFSA when assessing maltodextrin use as an insecticide (EFSA, 2013).
310 Although some reports have described the ability of extracts from *Stevia rebaudiana*
311 (Bertoni) to control fungal growth and mycotoxins production (Garcia et al., 2012), we

312 have not observed this effect when using purified steviol glycosides. However, the
313 proportion of steviol glycosides present in the products used in this study ranged from
314 1-2%, which might be far lower than concentrations used in the trials by Garcia et al.
315 (2012) and this may explain their results. It may not be economic at higher
316 concentrations in terms of commercial use. However, fungal spoilage issues may not
317 have been considered when developing these products as substitutes for sucrose in
318 bakery products.

319

320 In the reference cake containing 100% sucrose was an excellent hurdle to inhibit and
321 control the initiation of growth of these spoilage fungi, especially *E. amstelodami*, *A.*
322 *flavus* and *P. verrucosum*. At the lower temperature it was able to completely inhibit
323 all the test species. However, S1 product was unable to stop growth of *E. amstelodami*
324 and *P. verrucosum*. The partial substitution of sugar made the cake more susceptible
325 to fungal spoilage. Only the *F. graminearum* strain used was completely inhibited by
326 this product at both temperatures.

327

328 The shelf-life time prior to initiation of microscopic or visible moulding is critical for
329 bakery products. Sucrose and the S1 product, which included erythritol as a bulking
330 agent, were effective in controlling initiation of growth for up to 30 days. However, S2
331 and S3 were much less effective with very short shelf-lives prior to visible spoilage
332 becoming visible. This would suggest that unless combined with a range of other
333 hurdles such as preservatives or modified atmosphere packaging, products using
334 these two sugar substitutes would be prone to rapid fungal spoilage (Guenot et al.,
335 2005).

336

337 **Conclusions**

338

339 This study suggests that commercial Stevia sugar substitute products alone may not
340 be effective at controlling growth of spoilage fungi in cake-type bakery products. This
341 needs to be taken into account despite the fact that Struck et al. (2014) obtained good
342 results in terms of bakery product quality when substituting sucrose with Stevia-based
343 products. This study suggests that modified bakery product formulations which

344 incorporate Stevia products to reduce the overall sugar content needs to take into
345 account potential shorter shelf-life issues with regard to fungal spoilage in such
346 intermediate moisture bakery products. This may increase the relative risk for shorter
347 shelf-life of such products. Perhaps the use of such products in home baking requires
348 some additional information for consumers in relation to storage conditions to
349 minimise the risks of fungal spoilage in the domestic environment.

350

351 **Acknowledgements**

352 The authors wish to thank the collaboration of Mr. Miguel Roman Gomez-Gonzalez
353 during the baking process and downstream preparation of the cake analogues
354 samples.

355

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418

419 Table 1: General specific information in the products label with regard to weight,
 420 composition manufacturer, use guidelines and energy
 421

Product	Weight of the package	Composition	Manufacturer dosage recommendations	Energy values per 100 g
S1	270 g	Bulking Agent: Erythritol Sweetener: Steviol Glycosides (1% Stevia Leaf Extract), Natural Flavourings	1/3 teaspoon sweetens like 1 teaspoon of sugar	0 kJ (0 kcal)
S2	75 g	Bulking Agent: Maltodextrin Sweetener: Steviol Glycosides (2%), Natural Flavourings	1 teaspoon (2 kcal) is equivalent in sweetness to one teaspoon of sugar (20 kcal)	1598 kJ (376 kcal)
S3	75g	Bulking Agent: Maltodextrin Sweetener: Steviol Glycosides (2%)	1 teaspoon is equivalent in sweetness to one teaspoon of sugar	1656kJ (390kcal)

422
 423
 424
 425

426 **Figure Legends**

427

428 Figure 1. Comparison of the effect of different solutions (0-180 g solute/ 100 ml of
429 water) of sucrose, glucose, glycerol, and Stevia-based products S1, S2 and S3 on the
430 water activity levels.

431

432 Figure 2. Effect of temperature on the lag phases (days) of *A. flavus*, *F. graminearum*,
433 *E. amstelodami* and *P. verrucosum* on the cake analogue slices over a 1 month
434 incubation period. *Denotes conditions at which the lag time was longer than the
435 duration of the experiment.

436

437 Figure 3. Effect of temperature on the growth rate (mm diameter/day) of *A. flavus*, *F.*
438 *graminearum*, *E. amstelodami* and *P. verrucosum* on the cake analogue slices over a 1
439 month incubation period.

440

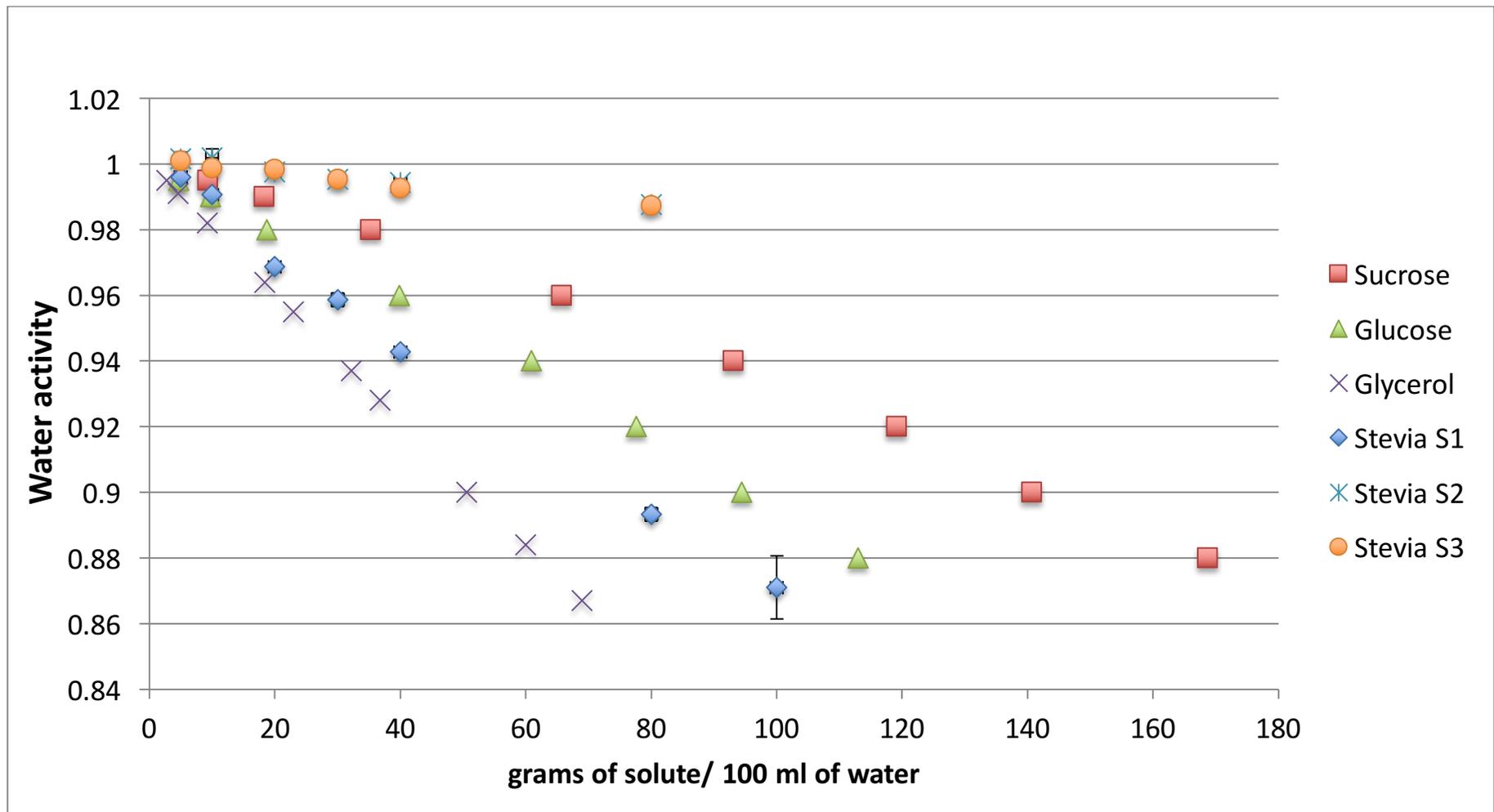
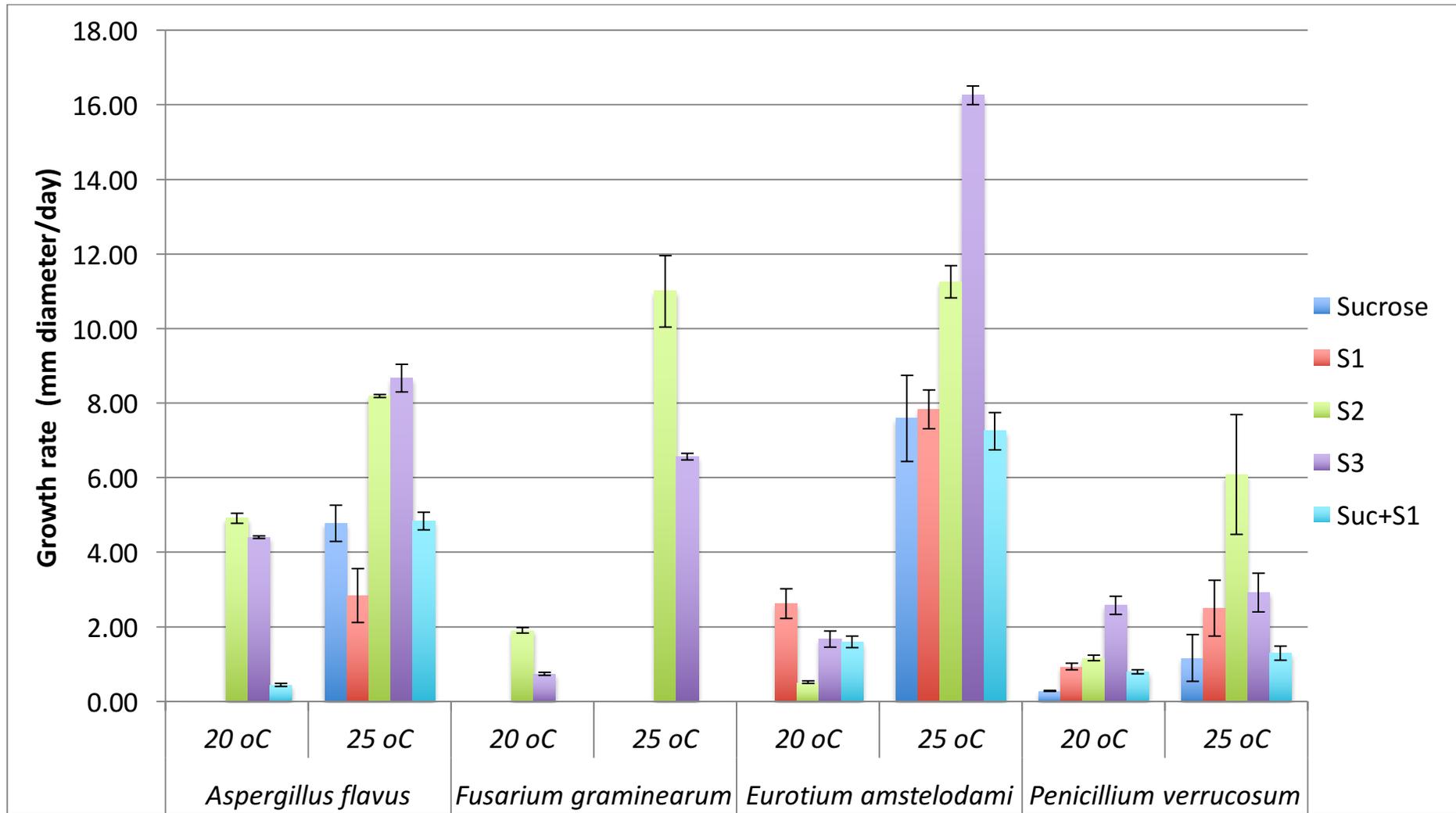
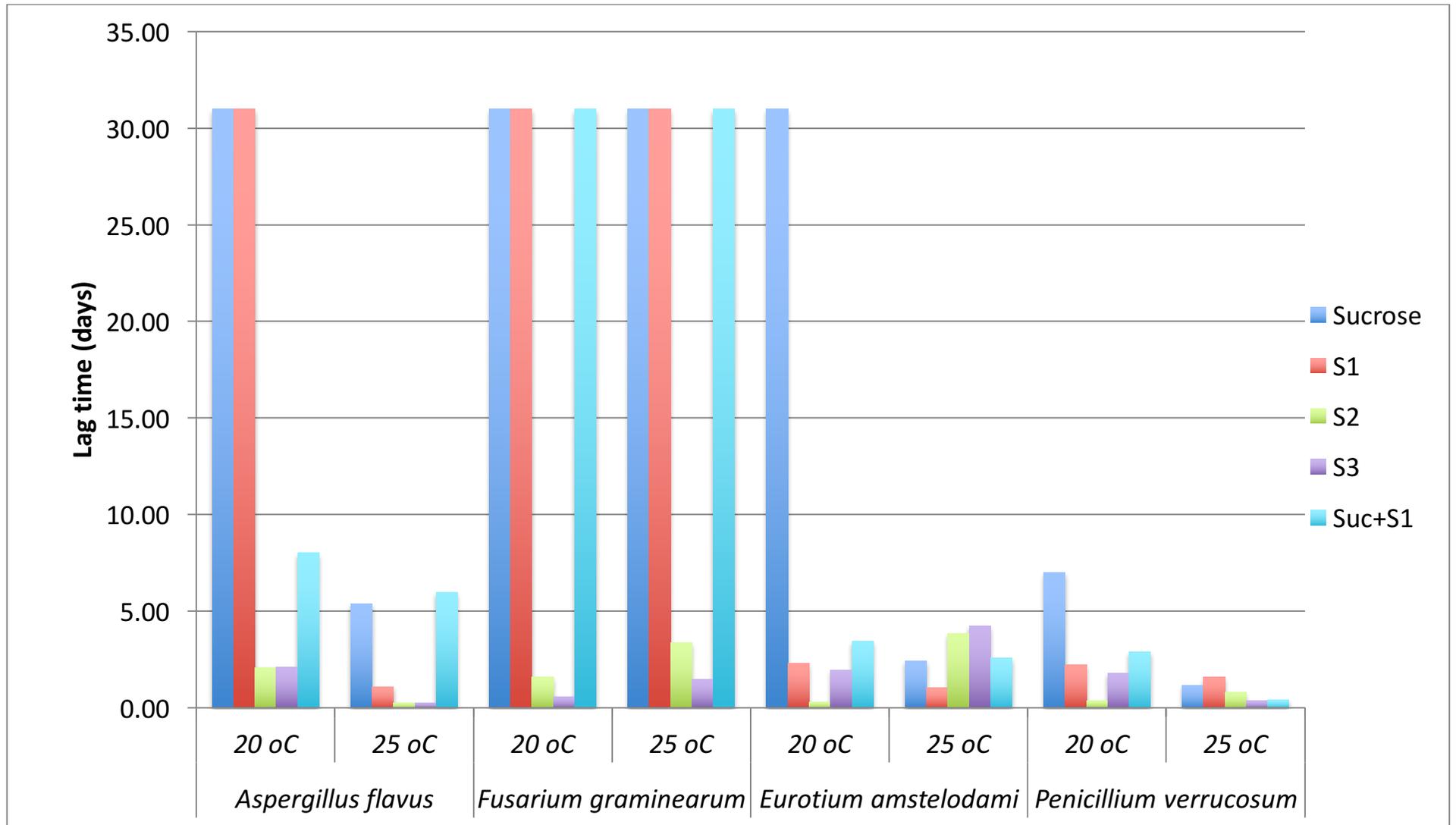


Figure 1 Rodriguez et al





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2016-04-27

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Alicia Rodríguez, Naresh Magan, Angel Medina, Evaluation of the risk of fungal spoilage when substituting sucrose with commercial purified Stevia glycosides in sweetened bakery products, International Journal of Food Microbiology, Volume 231, 16 August 2016, pp. 42-47

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