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#### 1 Development of a model for the alcoholic fermentation of cocoa beans by a Saccharomyces

#### 2 *cerevisiae* strain

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#### 8 Abstract:

9 The aromatic quality of chocolate requires the use of cocoa with high aromatic potential, this being 10 acquired during the fermentation of cocoa beans. Traditional fermentation is still often carried out on a small scale with wild strains of yeasts and acetic bacteria and under poorly controlled 11 conditions leading to cocoa quality ranging from best to worst. This study is the first part of a 12 project aiming to control quality of cocoa to produce high aromatic quality chocolate by using a 13 mixed starter of selected strains of yeast and acetic bacteria and by controlling the conditions of 14 fermentation. To achieve this objective, a mathematical model of the alcoholic fermentation of 15 cocoa beans has been developed. The growth, glucose consumption and ethanol production of 16 17 Saccharomyces cerevisiae LM strain in synthetic broth were modelled for the most important intrinsic (pH, glucose, ethanol, free nitrogen and oxygen levels) and extrinsic (temperature, oxygen 18 level) fermentation parameters. The model was developed by combining the effects of individual 19 20 conditions in a multiplicative way using the gamma concept. The model was validated in liquid synthetic medium at two different inoculation levels 10<sup>4</sup> and 10<sup>6</sup> CFU/mL with an increase in 21 temperature that recorded during spontaneous fermentations. The model clearly shows that the level 22 of inoculation and the speed of the increase in temperature clearly drive yeast growth, while other 23

factors including pH and ethanol, free nitrogen and oxygen levels have no significant impact onyeast growth.

26 Keywords: yeast; starter; chocolate; gamma concept; predictive microbiology

27

#### 28 **1. Introduction**

29 Flavor is the essential attribute of the quality of chocolate. It is directly linked to that of the raw cocoa beans that depends essentially on the post-harvest processing of beans, namely fermentation 30 and drying. Post-harvest processing is performed on-farm or in nearby small-scale processing units 31 and is poorly controlled leading to variable and/or poor quality cocoa beans (Aprotosoaie et al., 32 2016). Obtaining good quality marketable raw cocoa beans requires good agricultural practices 33 (selection of plants, selection of cocoa pods/beans, maintenance of fermentation equipment, 34 removal of the placenta) and good fermentation practices. Fermentation indeed plays a key role in 35 developing chocolate flavor precursors (Afoakwa et al., 2008; Beckett, 2003; Camu et al., 2008; 36 37 Owusu, 2010; Pereira et al., 2017; Ravelomanana et al., 1985; Schwan and Wheals, 2004). Before fermentation, cocoa beans are removed from the pod. They are surrounded by a white mucilaginous 38 pulp; rich in pectin, citric acid and sugars (Pettipher, 1986). Generally, in Côte d'Ivoire, the beans 39 40 are piled in heaps or placed in wooden boxes covered with banana leaves. The cocoa beans then begin to ferment. Fermentation occurs spontaneously and usually lasts from five to seven days. It 41 42 has two main phases. The first is anaerobic and lasts an average of two days during which yeasts 43 convert the sugars in the pulp into ethanol by alcoholic fermentation with an accompanying slight increase in the temperature that can reach 35 °C. At the same time, the pulp is hydrolyzed; this 44 allows the entry of air into the mass, creating aerobic conditions maintained by periodic turning of 45 the heaps of beans that favor the start of the second phase. During the second phase, oxygen is 46 consumed by acetic bacteria to oxidize ethanol into acetic acid, which can be over-oxidized to 47 48 carbon dioxide and water. As these reactions are exothermic, the mass temperature increases to

reach or even exceed 50 °C. At the same time, ethanol and acetic acid diffuse in the beans leading 49 50 to the death of the embryo but also to biochemical changes in the cotyledons such as the reduction of the sapid compounds responsible for bitterness and astringency, changing the color of the beans 51 and the production of the precursors of the chocolate flavor (Kadow et al., 2013; Trognitz et al., 52 2013). The presence of a large lactic population during the fermentation of cocoa beans has been 53 reported for many years. In addition, some authors identify a lactic phase, and lactic acid bacteria 54 would play a relevant positive or negative role. Overall, if the yeasts S. cerevisiae and H. opuntiae, 55 the lactic acid bacteria L. fermentum and the acetic acid bacteria A. pasteurianus lead the 56 fermentation, good marketable beans are generally obtained (Camu et al., 2008, 2007; Daniel et al., 57 58 2009; Nielsen et al., 2007; Papalexandratou et al., 2011; Papalexandratou and De Vuyst, 2011). For these authors, lactic acid bacteria by consuming more fructose than glucose and citric acid to 59 produce lactic acid, acetic acid, ethanol and mannitol would be essential for the proper progress of 60 61 acetic fermentation and the aromatic quality of fermented beans. However, for Gálvez et al. (2007) and Ho et al. (2015), lactic acid bacteria would compete for glucose with yeasts which would lead 62 to poorly ethanolic fermentation of beans and to beans with rich lactic acid content; this excessive 63 acidification can depreciate chocolate quality. 64

Inoculation with selected microorganisms has long been practiced to control the quality of 65 66 fermented foods including wine, beer and bread (Leroy and De Vuyst, 2004). Recently several authors tested selected starters for the fermentation of cocoa beans. Inoculated fermentation at 67 laboratory or pilot scale levels revealed the potential of this approach. The inoculation levels are 68 generally  $10^5$  or  $10^6$  CFU/g for yeasts. These levels lead to regular fermentation and the 69 development of desirable characteristics of the cocoa beans; higher inoculation levels would be 70 71 prohibitive for large-scale commercial use. Inoculated fermentation could also reduce the fermentation period to between three and five days instead of five to seven days for natural 72 fermentation (Lefeber et al., 2012; Meersman et al., 2015; Moreira et al., 2017; Sandhya et al., 73

2016; Schwan, 1998). The quality of the inoculated beans was generally better than the quality 74 75 obtained from spontaneous fermentation. Leal et al. (2008), during controlled fermentation of 45 kg cocoa beans inoculated with a strain of K. marxianus with high pectinolytic activity observed that 76 this strain was present throughout the fermentations characterized by a faster and more intense pulp 77 hydrolysis and a higher (+2°C) increase in temperature of beans mass. Inoculated cocoa beans 78 showed a higher level of cotyledonary storage proteins degradation, a lower level of titratable 79 80 acidity and a better cut-test score, indicating a higher proportion of well fermented beans. Sensorial analysis of the chocolate produced from cocoa beans fermented with K. marxianus gives better 81 results in terms of the flavor attributes and global acceptability. Crafack et al. (2013) used a mixed 82 starter culture composed of *P. kluyveri* or *K. marxianus* associated with *Lb. fermentum* and *A.* 83 *pasteurianus*. During fermentations of 20 kg of cocoa beans inoculated with a mixed starter a faster 84 consumption of citric acid was observed compared to spontaneous fermentation, whereas no major 85 86 differences in the pulp concentrations of acetic acid, lactic acid and mannitol were observed between the inoculated and spontaneous fermentations. After sensory analysis, chocolate produced 87 from inoculated beans was found to be sweeter, fruity and with a full cocoa flavor. However, tests 88 are still required at industrial scale to define the required conditions for inoculated fermentation as 89 the raw material is subject to great variability (the origin of the plants, selection of the cocoa 90 pods/beans, their maturity, the delay between pod opening and fermentation) and the environmental 91 conditions. Proper inoculation with different strains (at different levels and at different times) and 92 fermentation thus remain difficult to achieve without understanding the trophic competition and the 93 succession of microbial strains, and the impact of the changes in environmental conditions, 94 especially temperature, on the development of the strains. A modeling approach could help take all 95 these parameters into account and help control the fermentation process. 96

97 The use of tools and methods of predictive microbiology (Augustin, 2005) has enabled the 98 development of mathematical models of microbial growth under different environmental 99 conditions, including the fermentation of Gowé, by Mestres et al. (2017) and Munanga et al. (2016).
100 Modeling makes it possible to run simulations to evaluate the ability of the strains to control
101 fermentation, to define the relative rates of the different microbial species that are used in the
102 starter, the conditions of inoculation, and finally to define the parameters to control the process
103 including temperature, pH, and substrate concentration (Arellano-Plaza et al., 2007; Mestres et al.,
104 2017; Munanga et al., 2016).

105 This study was carried out as part of a project to control the production of premium chocolate characterized by floral and fruity notes. To achieve this goal, it was planned using a mixed starter 106 107 consisting of selected strains of yeast and acetic bacteria and to develop a global model of the cocoa 108 beans. The expected global model should be able to describe the growth, the use of substrates and the production of metabolites under various environmental conditions (temperature, pH, O<sub>2</sub>, organic 109 acids content and ethanol) of strains constituting the starter. The global model will result of 110 assembling two sub-models, that of alcoholic fermentation, and that of acetic fermentation. This 111 global model will be built progressively, step by step, by progressively assembling sub-models 112 113 recognized as being robust without their integration in the global model modifying the architecture of this one. The model of alcoholic fermentation developed here accounts for the most important 114 intrinsic (glucose, ethanol, and nitrogen contents, pH) and extrinsic (temperature, oxygen level) 115 fermentation parameters and can predict the growth of the yeast strain under the increasing 116 temperature observed during cocoa bean fermentation in real conditions. This model provides 117 insights into the parameters that drive the relay of the yeast population to that of acetic bacteria 118 observed during cocoa beans fermentation. 119

120

#### 2. Material and methods

121 **2.1.** Microbial strain

The *Saccharomyces cerevisiae* LM strain was provided by Lallemand S.A.S. (Blagnac, France)
as active dry yeast at a viable cell concentration of 10<sup>10</sup> CFU/g. The yeast was stored at 4 °C.
Before experiments, yeast was reactivated in sterile water at 35 °C for 30 minutes.

125

#### **2.2. Batch culture**

One liter Lambda Minifor fermenters equipped with a cold water condenser on air exit pipes. (LAMBDA Instruments GmbH, Baar-Switzerland), were used. Agitation, temperature, pO<sub>2</sub>, airflow, and pH were monitored and controlled. 1 M hydroxide sodium and 0.5 M sulfuric acid was used to adjust the pH. Yeast growth was monitored using an infrared in-line turbidity sensor (Optek FC20-ASD19-, Elscolab, Arcueil, France).

131

### 2.3. Microplate assays

Culture tests were carried out with a Bioscreen C MBR (Labsystems, Helsinki, Finland), in
sterile microplates under controlled stirring and temperature (30 °C). Turbidity was measured at
wavelength 600 nm. The growth rate was determined according to Membré et al. (2002).

135

### **2.4. Plate counting methods**

Tenfold serial dilution of samples (0.1 mL) was carried out in 9 g/L NaCl water and plated on
Sabouraud agar (Biokar-diagnostics, Beauvais, France). Plates were incubated at 30 °C for 48 h.
Counts are based on triplicate plating.

#### 139 **2.5.** Chemical analyses

Glucose, ethanol and glycerol contents were measured by HPLC (Dionex Ultimate 3000,
Thermo Electron SAS, Courtaboeuf, France) with separation on an Aminex HPX87H column
(Biorad, Hemel Hempstead, UK) and detection with both refractive-index and ultraviolet detectors
(210 nm). Elution was performed at 30 °C with 5 mM sulfuric acid at a flow rate of 0.6 mL/min.

144 **2.6. Modeling** 

#### 145 **2.6.1. Primary model**

146 The logistic model with delay and rupture (Rosso, 1995) was used to describe microbial147 growth, according to Equation (1).

148 
$$\frac{dNt}{dt} = \begin{cases} 0 , t \leq \lambda \\ \mu_{max} Nt \left(1 - \frac{Nt}{N_{max}}\right), t > \lambda \end{cases}$$
(1)

149 where  $\lambda$  is the lag phase (h),  $\mu_{max}$  the maximum specific growth rate (h<sup>-1</sup>), *Nt* and  $N_{max}$  (CFU/mL) the 150 microbial count at time t and at the stationary growth phase.

151 **2.6.2.** Modeling the lag phase  $(\lambda)$ 

In our conditions, i.e. direct inoculation with the *Saccharomyces cerevisiae* LM strain in the form of active yeast, the duration of the latency phase ( $\lambda$ ) in hours, increases linearly with temperature (T, °C) according to empirical Equation (2).

$$\lambda = a * T + b \tag{2}$$

#### 156 **2.6.3.** Secondary model

157 The gamma concept secondary model (Zwietering et al., 1993) was used to describe the impact 158 of temperature, pH, and glucose, ethanol, nitrogen content on the growth rate ( $\mu_{max}$ ), according to 159 Equation (3).

160 
$$\mu_{\text{max}} = \gamma(\text{T}^{\circ}\text{C}) * \gamma(\text{pH}) * \gamma([\text{Glucose}]) * \gamma([\text{Ethanol}]) * \gamma([\text{Nitrogen}])$$
(3)

161 with  $\gamma$  values ranging from 0 to 1.

### 162 *Impact of temperature and pH on yeast growth*

163 The values of gamma factor for temperature and pH, namely the cardinal temperature and pH 164 model (CTPM), proposed by (Rosso et al., 1995), were calculated according to equation (4)

$$165 \quad CTPM_{n}(X) = \begin{cases} 0, & , & X \leq X_{min} \\ \frac{(X - X_{max})(X - X_{min})^{n}}{(X_{opt} - X_{min})^{n-1} \{(X_{opt} - X_{min})(X - X_{opt}) - (X_{opt} - X_{max})[(n-1)X_{opt} + X_{min} - nX]\}} & , & X_{min} < X < X_{max} \end{cases}$$

$$(4)$$

where  $CTPM_n$  values range between 0 and 1. *X* corresponds to temperature or pH and the indices min, opt, max, to the minimum, optimum and maximum values for temperature or pH. n is a shape parameter that equals 1 for pH and 2 for temperature.

Temperature test cultures were performed in a one-liter glass fermenter using yeast nitrogen
base (YNB) without amino acids (Biokar-Diagnostics, Beauvais, France) supplemented with 20 g/L
glucose at a controlled pH of 4. Temperatures from 10 °C to 45 °C were tested.

pH test cultures were carried out Bioscreen C microplates according to Membré et al. (2002), in YNB without amino acids supplemented with 20 g/L glucose. The pH was adjusted from 3 to 8 (in progressive steps of 0.5) by adding sterile 1 M sodium hydroxide and 0.5 M sulfuric acid. The experimental temperature was set at 30 °C and all the tests were conducted in duplicate.

#### 176 *Effect of glucose*

In agreement with the substrate inhibition model of Andrews (1968), the value of the gammafactor for glucose and was calculated according to equation (5).

179 
$$\gamma([S]) = [S] / (Ks + [S] + [S]^2 / Ki)$$
 (5)

180 where  $\gamma([S])$  is the gamma factor of glucose ranging between 0 and 1, [S] is the initial glucose 181 concentration (g/L), *Ks* (g/L) is the saturation constant for glucose and *Ki*, the inhibition constant 182 (g/L).

183 Glucose culture assays were performed in sterile Bioscreen C microplates at pH 4 and 30 °C, in
184 YNB without amino acids, supplemented with concentrations of glucose ranging from 0 to 50 g/L.
185 Two tests were performed per glucose concentration.

#### 186 *Nitrogen content on yeast growth rate*

In agreement with the Monod model (Liu, 2006), the value of the gamma factor for nitrogenwas calculated according to equation (6).

189 
$$\gamma([S]) = [S] / (K_n + [S])$$
 (6)

189

where  $\gamma([S])$  is the gamma factor of nitrogen, ranging between 0 and 1, [S] is the initial nitrogen concentration (mg/L) and  $K_n$  (mg/L) is the saturation constant of the Monod model for nitrogen.

192 Nitrogen culture tests were carried out on sterile Bioscreen C microplates at pH 4 and 30  $^{\circ}$  C, in 193 YNB broth without amino acids and without ammonium sulfate added with 20 g / L of glucose and 194 nitrogen at concentrations ranging from 0 to 20 mg / L by adding di ammonium sulfate salt.

#### 195 Effect of ethanol content on yeast growth

196 The effect of ethanol on yeast growth was determined using equation (7) proposed by Coroller197 et al. (2005).

198 
$$\gamma([SI]) = 1 - \left(\frac{[SI]}{MIC}\right)$$
(7)

where [SI] is the ethanol concentration in g/L and *MIC* is the minimum inhibitory concentration ofethanol.

Ethanol culture assays were performed in sterile Bioscreen C microplates at pH 4 and 30 °C, using YNB broth without amino acids supplemented with 20 g/L of glucose and with ethanol concentrations ranging from 0 to 75 g/L, in duplicate.

#### 204 Kinetics of ethanol production

The kinetics of ethanol production was directly proportional to yeast growth by equation (8), using the model of Luedeking and Piret in which maintenance energy is disregarded (Van Impe et al., 2005).

208 
$$\frac{d[EtOh]}{dt} = Y_{(Etoh/N)} * \mu N$$
(8)

where [EtOH] is the concentration of ethanol (g/L) and  $Y_{(Etoh/N)}$  is the yield of ethanol in relation to 209 the yeast population (CFU/mL) and  $\mu$  is the specific growth rate (h<sup>-1</sup>). After integration, this 210 211 equation gives:

212 
$$[EtOH] = Y_{(Etoh/N)} * (N_t - N_0)$$
(9)

#### Where $N_0$ (CFU/mL) is the initial the population 213

#### 214 **Glucose** consumption

215 Similarly, glucose consumption is directly proportional to the yeast population and glucose content can be modeled by equation (10). 216

$$Glucose] = [Glucose]_0 - Y_{(glucose/N)} * (N_t - N_0)$$
(10)

where [Glucose]<sub>0</sub> is the initial glucose content and  $Y_{(glucose/N)}$ , is glucose consumption in relation 218 to the yeast population. 219

#### 2.7. Effect of oxygen 220

221 The effect of the oxygen concentration in the culture medium on the Saccharomyces cerevisiae LM strain growth was studied in one-liter Lambda glass fermenter containing YNB broth without 222 amino acid supplemented with 20 g/L glucose. The pH was set at 4 and the temperature at 30 °C. 223 Three levels of oxygenation (0, 4 and 8 mg/L) were tested. 224

225

## 2.8. Validation of the yeast growth model

The model was validated using the one-liter Lambda glass fermenter with YNB broth without 226 amino acid supplemented with 60 g/L glucose, at pH 4. A temperature profile (from 32 °C to 45 °C) 227 was programmed to follow as faithfully as possible the same increases in temperature as those 228 recorded during spontaneous semi-industrial fermentation of cocoa beans in Côte d'Ivoire (Fig. 1). 229 Two inoculation rates (10<sup>4</sup> or 10<sup>6</sup> CFU/mL) were tested in duplicate. Infrared absorbance was 230

231	monitored	throughout	fermentation	and	samples	were	collected	at	hourly	intervals	to	assess	the
		0							2				

232 metabolites.

237	Table 1: <mark>Parameters of the growth model in YNB medium taken into account</mark>
238	and environmental conditions.

Parameters	• $\mu_{opt}$					
	• $tlag(\lambda)$					
	• $T_{min}, T_{opt}, T_{max}$					
	• $pH_{min}$ , $pH_{opt}$ , $pH_{max}$					
	• <i>MIC</i> <sub><i>EtOH</i></sub>					
	• K <sub>s</sub> glucose					
	• Y <sub>gluc/CFU/mL</sub>					
	• Y <sub>ethanol</sub> /CFU/mL					
	• [Glucose] initial = $60 \text{ g/L}$					
Environmental conditions	<ul> <li><i>pH</i> = 4</li> <li><i>PO</i><sub>2</sub> = 0 mg/L</li> <li><i>Stirring</i> = 3 Hz</li> </ul>					
	• Temperature steps:					
	- from 0 h to 23 h : 32 °C to 36 °C					
	from $22 h$ to $42 h$ + $26 ^{\circ}C$ to $45^{\circ}C$					



Figure 1: Temperature programmed for validation tests during model validation (dashed curve). Temperatures recorded during spontaneous fermentation in an ivory wooden box Coast, (solid curve).

2.9. Data processing 247

The parameters of the models and their standard errors were adjusted with Origin® (OriginLab, 248 Northampton, MA, United States) software using linear or non-linear curve fitting with the 249 Levenberg and Marquardt iteration algorithm. Simulink® (MATLAB and Statistics Toolbox 250 Release 2012b, The MathWorks, Inc., Natick, Massachusetts, United States.) software was used to 251 252 build the global model of yeast growth.

253 3. Results and discussion

#### 3.1. Modeling growth kinetics on a synthetic medium 254

cerevisiae LM strain

Table 2 lists the values of growth kinetics parameters for the Saccharomyces cerevisiae LM 255 strain. The cardinal temperature values and pH were estimated by adjusting the cardinal model (Eq. 256 4) to the experimental data sets by minimizing the residual standard root mean square error 257 (RMSE). 258

Table 2: Secondary model parameter values ± standard errors for Saccharomyces

259

261

Modeled Variable	Equation	Parameter	Value		
		pHmin	$2.20 \pm 0.14$		
		pHmax	$7.92 \pm 0.19$		
		pHopt	$4.54 \pm 0.14$		
$\mu_{max}$ (h <sup>-1</sup> )	(4)	μopt	$0.46 \pm 0.01$		
		Tmin (°C)	$8.2 \pm 2.5$		
		Tmax (°C)	$41.8 \pm 0.3$		
		T opt (°C)	$33.3 \pm 0.5$		
<i>(</i> (h)	(2)	a (h.°C <sup>-1</sup> )	$-0.61 \pm 0.06$		
	(-)	b (h)	$27.5 \pm 1.8$		

MIC (g/L)	(7)	[Ethanol]	$92.2 \pm 3.8$
Ks (g/L)	(5)	[Glucose]	$0.27 \pm 0.02$
$K_i$ (g/L)			$209 \pm 43$
K <sub>n</sub> (mg/L)	(6)	[Nitrogen]	$0.04 \pm 0.01$
Y(ethanol/N) (mg/CFU)	(9)	Ethanol production (10 <sup>-7</sup> mg/CFU)	$1.59 \pm 0.08$
Y <sub>(glucose/N)</sub> (mg/CFU)	(10)	Glucose consumption (10 <sup>-7</sup> mg/CFU)	$3.34 \pm 0.10$
$\begin{bmatrix} 0.5 \\ 0.4 \\ \vdots \end{bmatrix} (a)$		$\begin{bmatrix} 0.5\\0.4\\ \hline 1 \end{bmatrix} \begin{bmatrix} 0.3\\ \hline 1 \end{bmatrix}$	b)
$\begin{bmatrix} 0.5 \\ 0.4 \\ -1 \\ -1 \\ -1 \\ -1 \\ -1 \\ -1 \\ -1 \\ -$	• • • • • • • • • • • • • • • • • • •	$\begin{bmatrix} 0.5 \\ 0.4 \\ - \\ - \\ - \\ - \\ - \\ - \\ - \\ - \\ - \\ $	b)
0.5 0.4 (a) (a) (a) (a) (a) (b) (c) (c) (c) (c) (c) (c) (c) (c) (c) (c			b)

- 271
- 272

Figure 2: Effect of the temperature at pH = 4 (a) and of the pH (b) at 30 °C on the maximum growth rate of Saccharomyces cerevisiae LM strain.

273 As shown in Figure 2, the CTPM secondary model fit the experimental data well (RMSE 0.04). The optimal growth temperature of the Saccharomyces cerevisiae LM strain was 33.3 °C, with  $\mu_{opt}$ 274 of 0.46 h<sup>-1</sup> (Fig. 2a). These values are in agreement with those in the literature. Walsh and Martin 275 (1977) reported a Topt range of 30 °C to 35 °C for S. cerevisiae and (Arroyo-López et al., 2009), 276 34.1 °C. According to Torija et al. (2003), the optimal growth temperatures for oenological yeast 277 strains are between 30 °C and 35 °C. The Saccharomyces cerevisiae LM strain appeared slightly 278 thermotolerant, with a  $T_{max}$  of 41.6 °C. In a comparative study of 27 strains of the genus 279 Saccharomyces and non-Saccharomyces, (Salvadó et al., 2011) reported that S. cerevisiae was the 280

most thermotolerant species with an optimum temperature close to 33 °C with a  $\mu_{max}$  of 0.45 h<sup>-1</sup> and 281 282 a maximum temperature of 45 °C. Daniel et al. (2009) isolated from fermenting cocoa beans 91 yeast strains able to grow between 25 °C and 35 °C, with the exception of a thermotolerant strain of 283 P. kudriavzevii predominant in all fermentations able to grow at 45 °C. The relative 284 thermotolerance of the Saccharomyces cerevisiae LM strain should allow it to dominate other 285 strains of yeast and more particularly non-Saccharomyces, as suggested by Salvadó et al. (2011) as 286 a temperature of 42 °C is usually reached after the beginning of the acetic fermentation. Indeed, 287 Meersman et al. (2015) selected thermotolerant hybrids (that can grow at 42 °C) which partly 288 contributed to their ability to dominate spontaneous pilot scale fermentations of cocoa beans. 289

The Saccharomyces cerevisiae LM strain appeared to be acid-tolerant with a  $pH_{opt}$  value of 4.5 290 and an almost constant maximum growth rate of 0.32 to 0.38 h<sup>-1</sup> with an increase in pH from 2.9 to 291 5.7. Absence of variation in the growth rate was also reported by for S. cerevisiae. Charoenchai et 292 293 al. (1998) showed that a pH between 3 and 4 did not significantly affect the growth rate and cell biomass of wine yeasts. At pH 6.5 and above, there was a marked decrease in the growth rate of the 294 Saccharomyces cerevisiae LM strain and at pH 8, growth stopped (Fig. 2b). During the alcoholic 295 fermentation of cocoa beans, the pH value is fairly stable, i.e. between 3.5 and 4, at the end of the 296 fermentation but sometimes reaches 5.5 (Gálvez et al., 2007). This suggests that pH has little effect 297 298 on the growth of the Saccharomyces cerevisiae LM strain during fermentation.

The *Ks* and *Ki* values for glucose are respectively of 0.27 g/L and 209 g/L were determined by adjusting the Andrew model (Eq. 5) to the experimental data (Fig. 3a). These results indicate that the growth of the *Saccharomyces cerevisiae* LM strain is very slightly impacted by the glucose concentration, which has been reported for glucose concentrations above 200 g/L by many authors including Charoenchai et al. (1998). Considering the low level of inhibition of the growth of the *Saccharomyces cerevisiae* LM strain for initial glucose concentrations close to 60 g / L, this effect will not be taken into account in the model. Considering the impact of the concentration of mineral nitrogen on the growth rate of *Saccharomyces cerevisiae* LM strain, *Kn* value for the Monod model (Eq. 6) was very low (fitted at 0.04 mg/L of free nitrogen, with a quite large uncertainty) (Fig. 3b). There are more free amino acids in pulp and their content increase from 1 to 5 mg/g upon fermentation (Camu et al., 2007). This shows that the amount of free nitrogen is not a limiting factor in the fermentation of cocoa beans and this parameter will consequently not be taken into account in the model.



Figure 3: Effect of the initial glucose concentration (a) and the nitrogen concentration (b)
on the growth rate of the Saccharomyces cerevisiae LM strain. Experimental data (black
dots) and fitted (dashed line).

316

312

317 Equation (7) satisfactorily describes the decrease in  $\mu_{max}$  with an increase in the ethanol 318 concentration (Fig. 4).



320





323







326



0.25

0.2

Figure 3: Inhibitory effect of ethanol on growth of the Saccharomyces cerevisiae LM strain. Experimental data (dots), fitted data (dashed line).

328	A <i>MIC</i> <sub>Ethanol</sub> of 92.5 g/L was calculated for the <i>Saccharomyces cerevisiae</i> LM strain at 30 °C
329	and pH 4. The Saccharomyces cerevisiae LM strain appears to be fairly resistant to ethanol. This
330	result is consistent with the values reported in the literature by Rose (1980) ranging from 55 g/L to
331	103 g/L, by Arroyo-López et al. (2010) values ranging from 95.6 to 141.4 g/L and by Antoce et al.
332	(1997) values ranging from 88.8 to 107.7 g/L. Samagaci et al. (2014) isolated 18 out of 36
333	pectinolytic strains isolated from cocoa able to grow at 30 $^{\circ}C$ at an ethanol concentration of 79 g/L
334	and 7 strains able to grow up to 94.8 g/L. The maximum concentration of ethanol is less than 20 g/L
335	(Camu et al., 2008, 2007; Lefeber et al., 2012; Schwan, 1998) during cocoa bean fermentation and
336	is in any case much lower than the MIC <sub>Ethanol</sub> of the Saccharomyces cerevisiae LM strain; the
337	ethanol concentration consequently has very little impact on the growth of this strain. Ethanol can
338	also have an antagonistic effect of temperature as high ethanol concentrations decrease the optimum
339	growth temperature of yeast strains and increase the lag phase (Medawar et al., 2003). Sá-Correia
340	and Van Uden (1983) reported that with an ethanol concentration close to $11\%$ (v/v) the maximum
341	growth temperature of a S. cerevisiae strain drops from 42 °C to 27 °C. Samagaci et al. (2014)
342	reported that 36 yeast pectinolytic strains isolated from fermented cocoa beans showing both
343	tolerance to alcohol (10-12% alcohol) and to temperature (up to 40 $^{\circ}$ C) failed to grow at only 35 $^{\circ}$ C
344	with less than 8-10% alcohol. The maximum level of ethanol recorded during cacao fermentation
345	being 5 to 10 times lower (less than 2%), the interaction between ethanol level and temperature can
346	be neglected in the model.

The value of the maximum growth rate was between 0.41 and 0.43  $h^{-1}$  (results not shown) for dissolved oxygen concentrations 0; 4 and 8 mg/L. This factor was thus not taken into account in the model.

Figure 5 shows the consumption of glucose and the production of ethanol by the Saccharomyces cerevisiae LM strain. The mass yield of ethanol from glucose (Yp/s) is 0.46 g/g, which is close to the theoretical value 0.51 g of ethanol per gram of glucose consumed. This theoretical yield is never reached as part of the glucose is used for the synthesis of biomass and secondary metabolites such as glycerol. In *S. cerevisiae* under oenological conditions, the observed yields are between 0.47 g/g and 0.48 g/g (Lonvaud et al., 2010). Similarly, Laopaiboon et al. (2007) found values ranging to 0.42 g/g to 0.48 g/g by fermenting sweet sorghum juice.





### 365

Figure 4: Relation between the concentration of glucose  $(\bullet)$ , ethanol  $(\blacktriangle)$  and the yeast population size.

#### **366 3.1.Validation of the model on synthetic medium**

The global alcoholic fermentation model was developed using the values of the parameters and 367 environmental conditions listed in Table 1. During the beans fermentation, some amount of 368 glucose is also consumed by lactic acid bacteria, this is not included in this model, only built to 369 predict the behavior of the yeast strain. The Saccharomyces cerevisiae LM strain was cultured 370 in yeast nitrogen base without amino acids, supplemented with 60 g/L glucose at pH 4, in 371 conditions approaching those observed at the beginning of cocoa bean fermentations 372 characterized by absence of sucrose, but an equal amount of glucose and fructose (Ho et al., 373 2015; Papalexandratou et al., 2011). We only added glucose as the *Saccharomyces cerevisiae* 374 LM strain can grow indifferently on glucose or fructose (results not shown). The temperature 375 376 was regulated to mimic the measured profile observed in a wooden box during spontaneous fermentation in Côte d'Ivoire (Fig. 1) and close to the temperature profile observed in Ghana 377 and the Dominican Republic (Camu et al., 2007; Gálvez et al., 2007; Pettipher, 1986). Two 378 levels of inoculation, 10<sup>4</sup> and 10<sup>6</sup> CFU/mL, were tested to assess the influence of the initial 379 yeast population on the course of the fermentation, given that this range is often used for the 380

inoculation of cocoa beans (Meersman et al., 2015; Ramos et al., 2014). In comparison, the
initial populations in natural cocoa fermentations are generally between 10<sup>3</sup> and 10<sup>7</sup> CFU/mL
(Ardhana and Fleet, 2003; Batista et al., 2015; Camu et al., 2007; Nielsen et al., 2007). It is
indeed likely that pod ripeness and postharvest pod age (due to transport and temporary
storage) determine the initial amounts of yeasts and LAB (Camu et al., 2007).

Figure 6 compares the experimental population measured during duplicated fermentation with

the population predicted by the model under the same conditions.

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Figure 5: Saccharomyces cerevisiae LM strain growth ( $\circ$  rep1 and  $\bullet$  rep2); simulated growth of yeasts (- - -); temperature profile (-.-.-) and  $\gamma$ -temperature (—) with inoculation level at 10<sup>4</sup> CFU/mL (a) and 10<sup>6</sup> CFU/mL (b).

The model predicted a lag phase of nearly 7 h for both fermentations whereas it was longer, 392 close to 10 h with an inoculation level of 10<sup>4</sup> CFU/mL and shorter, close to 3 h with an inoculation 393 level of 10<sup>6</sup> CFU/mL. The lag phase is indeed always difficult to predict (Augustin and Carlier, 394 2000; Medawar et al., 2003; Munanga et al., 2016) and the model presented a RSE of 2.1 h for the 395 396 lag phase. This difficulty in measuring and predicting the lag phase has several possible explanations, the heterogeneity of the population, the balance between the individual latency of 397 each cell and the latency of the global population, or the phenomena of intermediate latency 398 explained by Swinnen et al. (2004). Nevertheless, the differences between the predicted and 399 400 experimental populations are less than one log unit, and one can thus consider that the model

401 predicts the growth of the *Saccharomyces cerevisiae* LM strain fairly well. However, the model402 could be improved by a better determination of the lag phase.

With an inoculation level of  $10^4$  CFU / mL, the population increased for approximately 23 h 403 and reached a stationary phase of 33 h, while the model predicts growth arrest at 33.6 h when the 404 temperature reached 41 °C, i.e. the  $T_{max}$  of the Saccharomyces cerevisiae LM strain and when the  $\gamma$ -405 temperature became null (Fig. 6a). These results are consistent with those of Mensonides et al. 406 407 (2002), who showed that increasing the culture temperature of a strain of S. cerevisiae from 28 °C to 43 °C resulted in a sharp decrease in the growth rate at 40 °C, a complete end to growth at 42 °C, 408 and total loss of viability above 43 °C. On the other hand, with an inoculation level of 10<sup>6</sup> CFU/mL. 409 the growth of the population stopped after 21 h according to the model and at 26 h in the 410 411 experiment when the temperature was close to 37.5 °C, which corresponds to a *y-temperature* of 0.7 (Fig. 6b). Consequently, another parameter was responsible for stopping growth. 412

Figure 7 shows the changes in glucose concentration predicted by the model and those observed during the growth of the yeast strain. With an inoculation level of 10<sup>4</sup> CFU/mL, the model predicted changes in the glucose concentration fairly well up to 33 h when the temperature reached 416 41 °C. While the model predicted glucose consumption would stop at that time, in the experiment, 417 the consumption of glucose continued after 33 h and was only complete after 48 h (Fig. 7a).





Figure 7: Changes in ethanol concentrations ( $\Delta$  rep1 and  $\blacktriangle$ rep 2); simulated changes in ethanol (- - -) and  $\gamma$ -ethanol (--) during fermentation with inoculation levels of 10<sup>4</sup> CFU/mL (a) and 10<sup>6</sup> CFU/mL (b).

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The model predicts yeast growth, glucose consumption and ethanol production fairly well, 448 449 except at the end of the exponential phase. This is particularly true with the lower inoculation level when the temperature reaches  $T_{max}$  of the strain. This can be attributed to the fact that we did not 450 451 take decoupling between the production of ethanol and the consumption of glucose into account in the model, which reduces the yield of biomass and ethanol. This phenomenon is widely reported in 452 the literature as being the result of the adaptation of yeast to heat stress; as a consequence, the 453 energy required by the cell for maintenance increases due to the production of heat shock proteins 454 and metabolites such as trehalose and glycerol (Aldiguier et al., 2004; Kukec et al., 2003; 455 Mensonides et al., 2014; Omori et al., 1996). The concentrations of glycerol measured after total 456 glucose consumption were 4.15 g/L and 4.76 g/L respectively for inoculation levels of  $10^4$  and  $10^6$ 457 CFU/mL, i.e. nearly one quarter of the total amount ethanol produced. The ratio of glycerol 458 produced to glucose consumed (60 g/L) was between 0.069 g/g and 0.079 g/g. This ratio is higher 459 than that calculated for non-botrytized wines in which between 4 and 9 g/L of glycerol is produced 460 from an initial total sugar contents in grape juice of 200 g/L giving a ratio of between 0.02 g/g and 461

462 0.045 g/g (Remize et al., 2000). To improve the accuracy of the model, it thus appears necessary to 463 take into account the maintenance energy and the production of glycerol, particularly when the 464 model temperature increases. This is all the more important as, according to Illeghems et al. (2016), 465 glycerol could allow the growth of *A. pasteurianus* strains often found during the acetic 466 fermentation of the beans.

The model and the validation sets, performed in conditions that mimic cocoa fermentation, 467 clearly demonstrate that the inoculation level and the speed of increase of temperature drive the 468 yeast growth while other factors such as pH and ethanol, free nitrogen and oxygen levels do not 469 have a significant impact on yeast growth. If the inoculation level is high and/or the increase in 470 471 temperature is low, glucose is completely exhausted after 1 day, while with a low yeast inoculation level and/or a rapid increase in temperature, yeast stops growing before the glucose is exhausted. 472 Similar observations have been made for cocoa fermentation, i.e. complete exhaustion of glucose 473 474 after high level of yeast inoculation and 1-2 day fermentation has been reported (Crafack et al., 2013; Lefeber et al., 2012; Sandhya et al., 2016) while residual glucose has been reported after 475 476 natural fermentation or with a low level of yeast inoculation. The former leads to high transient ethanol content during cocoa fermentation, but to acidic, astringent chocolate with a quite low 477 hedonic rating (Sandhya et al., 2016) while the latter can lead to chocolate with a high hedonic rate. 478 479 In any case, the death of yeasts at the end of the anaerobic phase is largely documented (De Vuyst and Weckx, 2016) and attributed to depletion of fermentable sugar, an increase in temperature and 480 in pH and/or ethanol inhibition (Crafack et al., 2013; De Vuyst and Weckx, 2016). The model 481 developed here and the validation tests confirmed that the increase in the concentration of ethanol 482 and the change in pH do not have a significant impact on yeast growth or death during cocoa 483 fermentation, since most yeasts showed the same behavior as the Saccharomyces cerevisiae LM 484 strain. The increase in temperature and glucose exhaustion thus clearly drive yeast growth and death 485 during the anaerobic phase of cocoa fermentation. It should be noted that we did not observe yeast 486

death as the yeast population was calculated from measured absorbance and this increases with 487 488 biomass whether the biomass is alive or dead. As already mentioned above, maintenance metabolism and energy consumption during the stationary phase were not included in the model, 489 neither was yeast adaptation or the kinetics of death depending of the speed of the temperature 490 increase. Taking these phenomena into account could improve the predictability of the model, 491 particularly the consumption of glucose at the end of the growth phase when temperature is the 492 493 limiting growth factor, for example, with low inoculation levels. This work is a first step for developing a global model of cocoa pulp fermentation that can help for controlling cocoa 494 fermentation. The development of the model of alcoholic fermentation of the pulp surrounding 495 496 cocoa beans by Saccharomyces cerevisiae LM strain was based on the use of predictive microbiology models and particularly by using the gamma concept. The next step is under 497 development; a similar approach is being used to model acetic fermentation using a strain of acetic 498 499 bacteria isolated from an industrial fermentation in Côte d'Ivoire. Once validated, it will be combined with the alcoholic fermentation model to obtain the overall model of cocoa bean 500 501 fermentation. It will thus be validated at a laboratory scale, with a pilot scale fermenter, and on an 502 industrial scale in a fermentation center in Côte d'Ivoire. In addition, to take into account the consumption of the hexoses by LAB strains, an existing model of the lactic fermentation (Mestres 503 504 et al., 2017; Munanga et al., 2016) can be integrated into the global model of the fermentation of 505 cacao.

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#### Conclusion

507 This study is the first step of development of a global model of the cocoa beans fermentation of 508 a project whose goal is to control the aromatic quality of chocolate by using a mixed microbial 509 starter under controlled fermentation conditions. A model of alcoholic fermentation of the pulp 510 surrounding cocoa beans by *Saccharomyces cerevisiae* LM strain, based on the use of the gamma 511 concept, has been developed. Simulations and validations tests on a laboratory scale carried out with the model during fermentations under conditions mimicking those recorded during industrial fermentations in Côte d'Ivoire reveal the potential of modeling for the control of the anaerobic phase of cocoa fermentation. The model clearly show that the level of inoculation and the speed of the increase in temperature clearly drive yeast growth, while other factors including pH and ethanol, free nitrogen and oxygen levels have no significant impact on yeast growth.

The next step is developing, with a similar approach, a model acetic fermentation using a strain of acetic bacteria isolated from an industrial fermentation in Côte d'Ivoire and to combine both models to design a global model of the fermentation of the pulp of cocoa for predicting the evolution of substrates (glucose, fructose, ethanol) and metabolites (ethanol, acetic acid) that can have a direct impact of cocoa quality.

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