Modeling mixed fermentation of gowé using selected Lactobacillus plantarum and Pichia kluyveri strains

Christian Mestres, Bettencourt de J.C. Munanga, Joël Grabulos, Gérard Loiseau

Modeling mixed fermentation of *Gowé* using selected *Lactobacillus plantarum* and *Pichia kluyveri* strains

Christian MESTRES[1*], Bettencourt de J. C. MUNANGA[1,2], Joël GRABULOS[1], Gérard LOISEAU[3].

1 CIRAD, UMR Qualisud, F-34398 Montpellier, France.
Qualisud, Univ Montpellier, CIRAD, Montpellier SupAgro, Université d'Avignon, Université de La Réunion, Montpellier, France.

2 Instituto Superior de TecnologiaAgro-Alimentar de Malanje – ISTAM, EN. 230, Cangambo, Malanje -Angola ; kudymwena@yahoo.com.br

3 Qualisud, CIRAD, Montpellier SupAgro, Univ Avignon, Univ Guyane, Univ Montpellier, Univ Réunion, Montpellier, France

* Correspondance: Christian.mestres@cirad.fr

Abstract

This paper describes a mixed fermentation model made by assembling block hosting models for the growth of lactic acid bacteria (*Lactobacillus plantarum*) and a yeast strain (*Pichia kluyveri*), metabolic production and the physical-chemical changes which occur during the fermentation of *gowé*. The growth model for *P. kluyveri* was developed on a synthetic medium following the gamma concept taking into account the effect of pH, temperature, concentrations in glucose, lactic acid and ethanol. Additional parameters for the previously defined *L. plantarum* growth model were also determined (glucose and ethanol concentrations). The model was validated in three different *gowé* processing conditions. Even if the model underestimates LAB growth, it explains what occurs in the product and enables *in silico* extrapolation to various fermentation conditions. The predicted hydrolysis rates of native and gelatinised starches showed that increasing malt content is not an efficient way to increase the sweetness of *gowé* in contrast to increasing the level of pre-cooking. The builing-block model developed in this study could be applied to many other fermented foods and particularly to non-alcoholic but acid and sweet cereal based beverages.

Key words: Fermentation, Starter, Modelling, *Lactobacillus*, *Pichia kluyveri*, Saccharification.

1. Introduction

Gowé is an indigenous sorghum based sour beverage produced by spontaneous fermentation typically resulting from the growth of mixed cultures of lactic acid bacteria (LAB) and yeasts (Adinsi *et al.,* 2015; Akissoé *et al.,* 2015). Mixed fermentation occurs in many fermented food in addition to *gowé* such as sour bread, non-alcoholic but acid and sweet beverages like boza made from millet in Eastern Europe and Turkey, mahewu from maize in southern Africa (Blandino *et al.,* 2003) or cacao. It involve more than one microbial strain in interaction with others. Many different types of interactions are possible between microbial populations, but commensalism or mutualism is often observed when yeasts and lactic acid bacteria are involved in mixed fermentation. Yeasts can synthesise and release substances including vitamins, amino acids and purines, and break down complex carbohydrates, which are essential for the growth of lactobacilli species (Arroyo-López *et al*., 2008). Yeast species such as *Debaryomyces hansenii* and *Yarrowia lipolytica* assimilate the lactic acid formed by lactic acid bacteria, raising the pH and stimulating bacterial growth. In return, LAB produce lactic acid that lowers the pH, either inhibiting the growth of undesirable pathogens and/or promoting yeast growth (Nout *et al.*, 1989; Nout, 1991; Viljoen, 2006).

Two types of mathematical models are used to study the behaviour of a mixed fermentation; those only based on the growth curves of the species concerned and those which account for the mechanisms underlying the interaction (decrease in pH, consumption of limiting substrate or production of an inhibitory metabolite) (Cornu *et al*., 2011). In the first type of models, one (for the Jameson-effect) or two inhibition functions (for the Lotka-Volterra competition model) are adjusted; the model is thus simple and can be used in many cases but may be too simple to be applicable in all cases. These models do not provide any additional information on what occurs in the product (pH and biochemical changes). In the second type of models, pH and biochemical changes are explicitly modeled and are used to predict growth curves. These models are less parsimonious, but can be used to understand which parameters drive growth interactions and the modifications in food quality which occur during mixed fermentation. In previous articles, we proposed a model of the fermentation of *gowé* using a single starter culture made of lactic acid bacteria (Munanga *et al.,* 2016) and a second model of cereal starch hydrolysis during simultaneous saccharification and lactic acid fermentation of *gowé* (Mestres *et al*, 2017). The objective of the present article is to present an overall model of the fermentation of *gowé* which includes the previously cited models plus a growth model of the *Pichia kluyveri* strain.

2. Material and methods

2.1. Raw material

Grains of red sorghum [*Sorghum bicolor*, (L.) Moench], which are traditionally used to prepare *gowé*, were purchased on the local market in Cotonou (Benin). Malting was performed in the laboratory by steeping grains at 30 °C for 15 h followed by germination in an air-conditioned cabinet (30 °C, 98% relative humidity) for 72 h as detailed in Mestres *et al*. (2015). Raw sorghum and malted grains were ground in a Perten 3100 Laboratory Mill (Perten Instruments Hagersten, Sweden) equipped with a 0.5 mm sieve. Malted and non-malted sorghum flours were treated by gamma irradiation with 2 kGy (Ionisos Company, Danieux, France). The residual yeast population was less than 10^2 CFU/g and the residual population of lactic acid bacteria was below the threshold of detection.

2.2. Microbial strains

Frozen yeast, *Pichia kluyveri* strain (VINIFLORA® FROOTZEN®), was provided by Chr. Hansen Company (Prades-le-Lez, France) and stored at -80 ºC. *Lactobacillus plantarum* (CNCMI-3069) was provided by Ennolys (Soustons, France) as dry active bacteria stored at 4 °C. For all experiments, dry active bacteria were inoculated directly.

2.3. Microbiological methods

2.3.1. Pre-culture of yeast strain

The *P. kluyveri* strain was pre-cultivated by inoculating 1 g of the frozen strain in 50 mL of malt extract medium (Biokar-diagnostics, Beauvais, France) and incubated at 30 ºC for 24 h to reach an absorbance of 2.2 at 600 nm corresponding to a population of 10^7 CFU/mL.

2.3.2. Batch cultures

Six hundred milliliter glass double wall fermenters were used as described previously (Munanga *et al.,* 2016); turbidity was registred using an in-line near infrared sensor and cell count calculated according a calibration curve. The detection limit was of 0.01 which corresponds to a population level of $4 \cdot 10^5$ CFU/mL.

The effect of pH on the growth rate of *P. kluyveri* was determined by monitoring its growth rate on malt extract medium with a pH set between 2.5 and 9 at a fixed temperature of 30 ºC, and the effect of temperature was determined by setting the temperature at between 2 ºC and 45 ºC with a fixed pH of 6.5.

96 **2.3.3. Microplate assays**

97 Culture tests were carried out with a Bioscreen C MBR (Labsystems, Helsinki, Finland), in
98 sterile microplates under controlled stirring and temperature (30 °C, for yeast strain and 37 °C,
99 for the LAB strain). Turbidity was measured at 600 nm. Five replicates were performed for
100 each condition.

101 For *P. kluyveri*, YEPG broth buffered at pH 6 (1M phosphate buffer) was used for the
102 determination of minimum inhibitory concentration (MIC) for ethanol (added concentration
103 ranging from 0 to 8% v/v, i.e. up to 63 $g \cdot L^{-1}$) and for lactic acid (added concentration ranging
104 from 0 to 25 $g \cdot L^{-1}$). Ks (half-velocity constant) and Ki (inhibition constant) for glucose were
105 determined with YEP by adding glucose from 0 to 50 $g \cdot L^{-1}$. The initial yeast population was of
106 10^6 CFU/mL.

107 For *L. plantarum*, the MIC of ethanol was determined in MRS broth with ethanol concentrations
108 ranging from 0 to 8% (v/v) (i.e. up to 63 $g \cdot L^{-1}$). The Ks for glucose was determined with YEP
109 by adding glucose from 0 to 50 $g \cdot L^{-1}$.

110 **2.3.4. Plate methods**

111 Samples were serial diluted ten times (0.1 mL) in 9 $g \cdot L^{-1}$ NaCl water and plated on de Man
112 Rogosa and Sharpe agar (MRS) with pH adjusted at 6.2 for LAB and on Sabouraud
113 chloramphenicol agar medium (Biokar-diagnostics, Beauvais, France) for yeasts. MRS plates
114 were incubated at 37 ºC for 48 h and Sabouraud plates were incubated at 30 ºC for 48 h before
115 counting.

116 **2.3.5. Validation tests for *P. Kluyveri***

117 Validation tests were performed by monitoring batch cultures at 30 °C with no pH control first
118 on the malt extract medium then on *gowé* prepared at laboratory scale as previously described
119 (Munanga *et al.,* 2016) after inoculation at 10^6 CFU/mL. Samples were collected at hourly
120 intervals to measure the substrate and the concentration of products and to enumerate microbial
121 populations.

122 **2.3.6. Validation tests with mixed inoculation with *P. Kluyveri* and *Lactobacillus***
123 ***plantarum***

124 Validation tests with mixed inoculation were also performed on *gowé* prepared in the
125 laboratory. The same procedure as above was used with the modifications listed in Table 1.

126

Table 1. Conditions for the validation tests (V1-V3) and simulated fermentation (S1-S2) of *gowé*

Experiment	Pre-cooked flour (g)	Pre-cooking temperature (ºC)	Uncooked flour (g)	Malt flour (g)	Inoculation rate (CFU/g)		Fermentation temperature (ºC)
					Lactobacillus plantarum	*Pichia kluyveri*	
V1	15	80	60	25	$7.3 \cdot 10^5$	$7.0 \cdot 10^5$	25
V2	15	80	60	25	$5.4 \cdot 10^6$	$1.8 \cdot 10^4$	35
V3	20	80	55	25	$5.8 \cdot 10^5$	$6.3 \cdot 10^4$	40
S1	30	85	45	25	$5.0 \cdot 10^5$	$5.0 \cdot 10^5$	40
S2	30	85	45	25	$1.0 \cdot 10^6$	$1.0 \cdot 10^5$	40

2.4. Chemical Analysis

Lactic acid, glucose, maltose and ethanol contents were measured by HPLC as previously described (Munanga *et al.,* 2016).

2.5. Mathematical modelling

2.5.1. Primary and secondary models

The logistic growth model with delay (Rosso, 1995) was used to describe microbial growth (Equation (1)).

$$\begin{cases} \frac{dNt}{dt} = 0 & if\, t \le \lambda \\ \frac{dNt}{dt} = \mu_{max}\, N_t \left(1 - \frac{N_t}{N_{max}}\right) & if\, t > \lambda \end{cases} \quad (1)$$

where N_t and N_{max} (CFU/mL) represent the microbial population at time t and at the end of the growth curve, respectively, μ_{max} the maximum growth rate (h^{-1}) and λ the lag time (h).

The gamma concept model (Zwietering *et al.*, 1993) was used as secondary model to describe the impact of temperature (T), pH, concentrations in lactic acid ([AL]), ethanol ([EtOH]) and glucose ([glucose]) on maximum growth rate (μ_{max}) (Equation (2)).

$$\mu_{max} = \mu_{opt} * \gamma(T) * \gamma(pH) * \gamma([AL]) * \gamma([EtOH]) * \gamma([glucose]) \quad (2)$$

with γ values between 0 and 1.

The effect of temperature and pH on μ_{max} was expressed using the cardinal temperature and pH model (CTPM) proposed by Rosso *et al.* (1995) according to Equation (3).

147 $$CMn(X) = \begin{cases} 0, & X \le X_{min} \\ \frac{(X-X_{min})^n (X-X_{max})}{(X_{opt}-X_{min})^{n-1}\{(X_{opt}-X_{min})(X-X_{opt})-(X-X_{max})[(n-1)X_{opt}+X_{min}-nX]\}}, & X_{min} < X < X_{max} \\ 0, & X \ge X_{max} \end{cases} \quad (3)$$

148 where X corresponds to environment factors (pH, temperature) and 'n' to a shape parameter
149 that equals 2 for temperature but was adjusted for pH.

150

151 **2.5.2. Modelling product formation and glucose consumption, and metabolite**
152 **interactions**

153 Equation (4) was used to model the formation of ethanol (dP/dt) by *P. kluyveri,* where $Y_{EtOH/N}$
154 is the yield of ethanol over *P. kluyveri* population and μ is the specific growth rate (h^{-1}) (Van
155 Impe *et al*., 2005):

156 $$\frac{\mathrm{d[EtOh]}}{\mathrm{dt}} = Y_{(Etoh/N)} * \mu N \quad (4)$$

157 After integration, this equation gives:

158 $$[EtOH] = Y_{(Etoh/N)} * N \quad (5)$$

159 The effect of the concentration of ethanol on *P. kluyveri* and LAB strain growth was determined
160 using Equation (6) proposed by Coroller *et al*. (2005):

161 $$\gamma([EtOH]) = 1 - \left(\frac{[EtOH]}{MIC}\right)^{\alpha} \quad (6)$$

162 where [EtOH] represents the ethanol concentration in % v/v, MIC the concentration of ethanol
163 for which growth is completely inhibited and α reflects the shape factor of the curve.

164 The MIC of lactic acid was calculated with Equation (6), the same as for ethanol.

165 Like ethanol production, glucose consumption is directly proportional to the yeast population
166 and glucose content can be modelled by Equation (7):

167 $$[\mathrm{Glucose}] = [\mathrm{Glucose}]_0 - Y_{(glucose/N)} * N \quad (7)$$

168 where $[\mathrm{Glucose}]_0$ is the intial glucose content and $Y_{(glucose/N)}$, is glucose consumption in
169 relation to the yeast population.

170 In agreement with the Monod model, the gamma value for glucose was calculated according to
171 Equation (8) for LAB:

172 $$\gamma([S] = \frac{[S]}{K_s+[S]} \quad (8)$$

where [S] is the substrate (glucose) concentration in $g \cdot L^{-1}$.

To take the inhibition of growth due to excess substrate into consideration, the model was modified according to Song *et al*. (2008) for *P. kluyveri* (Equation 9):

$$\gamma([S]) = \frac{[s]}{\left\{[S]+K_s+\left(\frac{[S]^2}{K_i}\right)\right\}} \quad (9)$$

.

2.5.3. Implementation of the mixed fermentation model

To model mixed fermentation, we applied the global model of fermentation of lactic acid bacteria and of starch hydrolysis proposed by Munanga *et al*. (2016) and Mestres *et al*. (2017), and added the models of the effects of the glucose and ethanol on LAB growth and the model developed for the growth of *P. kluyveri*. The models were implemented in Simulink (Mathworks Inc., Natick, Mass, USA).

2.6. Statistical methods

The confidence intervals of means (at 95% probability) for non-linear and linear regressions were calculated using XLstat (Addinsoft, Paris).The cardinal values of Equation. (3) were fitted by the Levenberg–Marquardt method using the macro bundle, as proposed by De Levie (2001).

3. Results and discussion

3.1. *P. kluyveri* growth model

The logistic model (Equation (1)) fitted the experimental data obtained for the growth of *P. kluyveri* in malt extract broth at the different temperature and pH conditions quite well (residual standard root mean square error, RMSE, was of 0.03 log CFU), as shown in Fig. 1. The cardinal temperature and pH were estimated (Table 2) by adjusting the cardinal model (Equation (3)) to the experimental data sets (pH ranging from 2.5 to 9.0 and temperature ranging from 2 °C to 45 °C; Fig. 2) Minimum and maximum cardinal values were determined experimentally and the value of the coefficient n for pH was set at 0.1, as it allowed the best adjustment of the parameters of the CTPM by minimising RMSE which was of 0.04.

202 Table 2: Parameters for the growth model of *P. kluyveri*

Modeled variable	Equation	Parameter	Value ± SD* (*Pichia kluyveri*)
μ_{max}	(3)	pH_{min} pH_{max} pH_{opt} n (pH) μ_{opt} (h^{-1}) T_{min} (°C) T_{max} (°C) T_{opt} (°C) λ (h)	2.4 9.0 4.3 (± 0.2) 0.1 0.73 (±0.03) -9.2 45 37.6 (± 0.6) 2.0 (1.1)
MIC	(5)	[LA] $g \cdot L^{-1}$	30.7 (±1.1)
MIC	(5)	[EtOH] % v/v	5.1 (±0.3)
Ethanol production rate	(4)	$Y_{Ethanol/N}$ (mg/CFU)	4.99 10^{-8} (±0.13)
Glucose consumption rate		$Y_{glucose/N}$ (mg/CFU)	1.40 10^{-7} (±0.058)
γ[glucose]	(7)	K_s ($g \cdot L^{-1}$) K_i ($g \cdot L^{-1}$)	0.68 (±0.11) 86 (±23)

203 *SD: Standard Deviation*

204 The μ_{opt} calculated for *P. kluyveri* was 0.73 ± 0.03 h^{-1} (Table 2) and the impact of pH on the
205 growth rate of *P. kluyveri* was very low, between 2.5 and 6.2, as reported for other yeast strains
206 in similar pH ranges (Betts *et al*., 1999; Praphailong and Fleet, 1997; Rouwenhorst *et al*., 1988)
207 It should be noted that, as the yeast was pre-cultivated, lag time did not vary significantly with
208 the growth conditions (pH and temperature), its mean value was 2.0 (h).

209 The MIC value of lactic acid for *P. kluyveri*, 30.7 $g \cdot L^{-1}$ (Table 2), was lower than the MIC value
210 (548 mM, i.e. 49.3 $g \cdot L^{-1}$) for a strain of *Saccharomyces cerevisiae* (Thomas *et al*., 2002).

211 The MIC value of ethanol for *P. kluyveri,* 5.03 ± 0.02 % (40 $g \cdot L^{-1}$, Table 2) was close to the
212 values cited by Du Preez *et al*. (1987) (4.6-4.8 %) for *Pichia stipitis* and *Candida shihatae*
213 strains. A slightly lower MIC value was observed by Delgenes *et al*. (1988) for *P. stipitis*.

214 A sharp increase in μ_{max} was observed (Fig. 3) with an increase in glucose concentration
215 followed by a slight decrease of 6-7 $g \cdot L^{-1}$. Ks and Ki were adjusted to 0.68 $g \cdot L^{-1}$ and 86 $g \cdot L^{-1}$,
216 respectively, which means that the maximum growth rate is reached with low levels of glucose
217 and remains relatively constant below 50 $g \cdot L^{-1}$ of glucose.

218 Fig. 4 shows the relationships between the concentrations of metabolites (glucose and ethanol)
219 and the microbial load measured in different fermentation (pH, temperature) conditions. The
220 ratio of glucose consumption and of ethanol production to the microbial load were independent

of the growing conditions, at $1.4 \cdot 10^{-7}$ mg/CFU and $4.99 \cdot 10^{-8}$ mg/CFU, respectively. The mass yield of ethanol from glucose ($Y_{p/s}$) was 0.36 $g \cdot g^{-1}$. This is quite low compared to the 0.51 $g \cdot g^{-1}$ theoretical value of glucose conversion to ethanol and to the 0.48 $g \cdot g^{-1}$ value observed for a *Saccharomyces cerevis*iae oenological strain (Lonvaud et *al*, 2010). No data for *P. kluyveri* strains have been published, however, we can mention a value of 0.26 $g \cdot g^{-1}$ for *P. stipitis* reported by Mussatto *et al.* (2012).

3.2. Complementary model for LAB

A model of LAB growth has been already developed (Munanga *et al*., 2016). In the case of a mixed culture, such as *P. kluyveri* and *L. plantarum*, the global model must take into account the impact of the glucose concentration, consumed competitively by both strains, and the impact of the ethanol produced by the yeast on the growth of the lactic strain.

A Ks value of 1.44 (± 0.03) $g \cdot L^{-1}$ was determined, which is it close to the values cited by Charalampopoulos et *al*. (2009) and Sharma and Mishra (2014) for *L. plantarum*, at 1.48 and 1.32 $g \cdot L^{-1}$, respectively. The ethanol MIC value, 6.29 ± 0.03 % (i.e. 50 $g \cdot L^{-1}$, with a shape factor of 2.8 ± 0.1), showed that *L. plantarum* strain displayed slightly higher tolerance to ethanol than *P. kluyveri*. Alegría *et al*. (2004) reported an even higher MIC value of ethanol (13 %) for *L. plantarum.*

3.3. Validation of the growth model of *P. kluyveri* strain

The global model of *P. kluyveri* was tested in pure culture in YEPG broth (Fig. 5) and at the manufacturing scale used for *gowé* (Fig. 6). In YEPG broth with no control of pH, the model fitted the experimental data for the yeast population (Fig. 5a), glucose consumption and ethanol production very well (Fig. 5b), with respective RMSE of 0.35 log CFU, 0.20 and 0.29 $g \cdot L^{-1}$. Fermentation stopped at a quite low population level ($4 \cdot 10^7$ CFU/g). The model showed this was due to the exhaustion of glucose while the maximum population in the primary model was set at a much higher level (10^{10} CFU/g). The pH decreased slightly from 4.7 to 4.1 during fermentation which could be linked to the production of acetic acid by *P. kluyveri*. This did not significantly affect the growth of the yeast; the pH gamma value remained between 0.99 and 0.94 when the pH varied from 4.7 to 4.1. The production of acetic acid was not taken into account in the global model because its level of production was too low to influence the final pH of the *gowé* when it was produced by a mixed (LAB and yeast) microbial population.

When the mixture of sorghum flour and malt (Fig. 6a) was inoculated with *P. kluyveri*, the evolution of the population was also well predicted by the model (RMSE of 0.23 log CFU) with, in particular, a final yeast population 10 times higher than that observed and predicted in YEP broth. Glucose and maltose content first increased for 8 h, as predicted by the model, linked to enzymic hydrolysis of starch. Glucose consumption by yeast increased with an increase in the population of *P. kluyveri* and, after 10 h, exceeded maltose and glucose release; glucose and maltose content thus began to decrease and became almost zero after 15 h, almost stopping yeast growth. The model indeed predicts residual growth (μmax between 0.02 and 0.03 h^{-1}) promoted by the quite low but continuous release of free sugars from starch hydrolysis. Ethanol production was also quite well predicted by the model (RMSE of 0.4 $g \cdot L^{-1}$); the gamma value for ethanol after 10 h was 0.90 and did not really affect growth.

In parallel, changes in pH showed two phases (Figure 6b); first a decrease from 5.9 to 5.4 within 10 h which could be linked to low production of acetic acid by the yeasts, followed by a sharper decrease to pH 4.1 probably linked to the growth of wild lactic bacteria present in the raw material used.

3.4. Validation of the global model on mixed fermentation

Different *gowé* manufacturing conditions involving *L. plantarum* and *P. kluyveri* were used to validate the global model of *gowé* fermentation and to test different conditions for the preparation of *gowé* (Table 1). Marked variability in the process has indeed been observed (Adinsi *et al*., 2014; Michodejehoun *et al.*, 2005; Vieira-Dalode *et al.,* 2007); sorghum malt may be the only raw material used, but usually the proportion of sorghum malt is 20-25%, the other raw matrial being raw sorghum or maize. In addition, one quarter (25%) of the raw material is often pre-cooked at 70-80 °C or even 100 °C. Initial LAB and yeast levels in traditional natural fermentation of *gowé* can vary from 10^4 to 10^6 CFU/g and from 10^3 to 10^6 CFU/g, respectively. Various conditions were evaluated once in order to explore the large variability in the processing conditions and to test the robustness of the model.

Fig. 7 shows the results of the first validation test (V1) performed in conditions close to those used in traditional processing with, in particular, a fermentation temperature close to ambient temperature and a similar inoculation level for both strains. The model fitted the experimental data for the microbial population well (Fig. 7a; RMSE of 0.25 and 0.48 log CFU for *P. kluyveri* and *L. plantarum*, respectively), with only slight underestimation of the lactic population. The

285 stationary growth phase began after about 22-25 h of fermentation. The model predicted the
286 experimental values of pH, lactic acid (Fig. 7b) and ethanol (Fig. 7c) contents quite well (RMSE
287 of 0.5 and 1.9 $g \cdot L^{-1}$, respectively). Changes in the concentration of sugars (glucose + maltose)
288 were roughly predicted by the model, with, in particular, an initial increase linked to dominant
289 amylase activity, followed by a decrease linked to increasing consumption by yeasts and lactic
290 bacteria. However, the sugar content was slightly underestimated by the model; the final
291 concentration of sugars at the end of the experiment was between 5 and 10 $g \cdot L^{-1}$ while the
292 model predicted their total consumption. Model underestimation was particularly high at the
293 beginning of fermentation; the sugar content of the dough at zero fermentation time was indeed
294 45 $g \cdot L^{-1}$ while the value calculated from the sugar contents in malt and in raw sorghum was 15
295 $g \cdot L^{-1}$. Low soluble α-amylase activity (0.1 U/g), naturally present in sorghum grain, cannot
296 explain starch hydrolysis that occurred during the pre-cooking step which was performed at a
297 temperature close to the optimum temperature for sorghum α-amylase (Mestres *et al.,* 2017); a
298 contribution of insoluble amylase may be assumed. After about 22-25 h of fermentation, a pH
299 of 4 was reached while free sugar content was low to zero; the former condition stopped the
300 amylase activity (Mestres *et al.,* 2017) while the latter stopped the growth of *L. plantarum* and
301 *P. kluyveri.* The driving gamma value was indeed that of sugar content which was predicted to
302 be zero after 24 h. The other gamma values remained over 0.6 except that of pH for *L. plantarum*
303 which dropped to 0.2 at 25 h. At the end of the fermentation process, a safe product (pH by 4.0)
304 with non-active LAB and yeasts and low alcohol content (20 $g \cdot L^{-1}$) was obtained. However, the
305 resulting *gowé* contained no sugars and will consequently not be appreciated by consumers
306 (Adinsi *et al.,* 2014, 2015)

307 Fig. 8 shows the results of the second validation test (V2) for which *L. plantarum* inoculation
308 level was three hundred times greater than that of *P. kluyveri* and the fermentation temperature
309 close to the optimum growth temperature of the two strains (Munanga *et al*., 2016). The model
310 clearly (Fig. 8) overestimates the lag phase and underestimates the LAB population and lactic
311 acid production, and consequently predicts a longer delay and a smaller drop in pH. The model
312 predicts a final population one log lower than the experimental value, and a very low growth
313 rate after 13.5-14 h. The low growth rate was linked to low gamma values of pH (0.15 at pH
314 3.9) and of undissociated lactic acid (0.15 at 13.5 h and dropping to 0 at 25 h) while gamma
315 values for glucose and ethanol were over 0.85. In contrast to the model, the experimental data
316 showed a high level of production of lactic acid after 15 h whose concentration reached more
317 than 16 $g \cdot L^{-1}$ after 24 h. This may be linked to the positive interactions between yeast and lactic

318 acid bacteria that were not taken into account in the global model. Such interactions have been
319 reported by many authors. Vitamins and co-factors accumulated and/or synthesized by yeasts
320 which promote the growth of lactobacilli include thiamine (vitamin B1), nicotinic acid,
321 pyridoxine (vitamin B6) and pantothenic acid (Arroyo -López *et al*., 2008). Gobbetti *et al.*
322 (1994) showed a commensalistic relationship between yeasts and lactobacilli of leaven bread
323 microbiota which benefited lactic bacteria, whose growth was more rapid in coculture with
324 yeasts. For populations of yeast, the stationary phase was reached after 15 h whereas the model
325 predicts continuous growth up to 25 h. Sugar and ethanol contents were reasonably predicted
326 by the model, although ethanol was slightly underestimated. Like in V1, the original sugar
327 content was underestimated, but the maximum value and the decrease after 10 h were well
328 predicted. It should be noted that this decrease is linked to the increasing sugar consumption by
329 the increasing microbial load but also to the inhibition of α-amylase at low pH; α-amylase was
330 predicted to be completely inhibited after 12 h, whereas it should have stopped after 10 h when
331 the pH reached 4 (Mestres *et al.,* 2017). To sum up, the fermentation of *gowé* in the conditions
332 in the V2 test could be stopped after 10-12 h, which would make the *gowé* sufficiently sour (pH
333 4.0), medium sweet (55-60 $g \cdot L^{-1}$) with low alcohol content (2-3 $g \cdot L^{-1}$).

334 For the V3 test, the quantity of pre-cooked flour was increased by 25%, the fermentation
335 temperature was slightly higher (but close to the T_{opt} of both strains) to promote starch
336 hydrolysis, and the inoculation level of *L. plantarum* was divided by 10. Like for V2, the model
337 (Fig. 9) overestimated the lag phase for LAB and consequently the beginning of the increase in
338 lactic acid production and the decrease in pH. The model predicted the lag phase and yeast
339 growth reasonably well (RMSE of 0.4 log CFU). The initial concentration of sugars was higher
340 than the concentrations measured in the other tests due to the increase in the quantity of
341 precooked flour used in this test. Like in the previous tests, the sugar content increased to a
342 maximum (by 70-80 $g \cdot L^{-1}$ after 14-15 h) then decreased due to the inhibition of α-amylase as
343 soon as pH dropped to below 4. The concentration of ethanol became significant only after 10
344 h and was predicted to increase up to 20 $g \cdot L^{-1}$ after 25 h. To sum up, 12 h of fermentation of
345 *gowé* in the conditions used for the V3 test would lead to a sufficiently sour, highly sweetened
346 product with a very low alcohol content.

347 The three validation tests provide some information about the robustness of the model. We
348 compared experimental and calculated values (Figure 10) and calculated the RMSE for the
349 duration of fermentation (i.e. how long it took to reach pH 4), level of maltose and glucose and
350 of ethanol at the end of fermentation. The RMSE were of 2.2 h, 15.7 $g \cdot L^{-1}$ and 0.9 $g \cdot L^{-1}$,

351 respectively. The agreement between the model and the obervations for the three validations
352 tests was thus satisfactory for the duration of fermentation and ethanol content at the end of the
353 fermentation. The model however slightly underestimated the level of maltose and glucose for
354 the three validation tests.

355 The validation tests and the model provide clear evidence that the main factors which control
356 the fermentation and the quality of *gowé* are the temperature of fermentation and the level of
357 pre-cooking. When the fermentation temperature was increased from 25 °C to 40 °C, the
358 duration of fermentation (i.e. how long it took to reach pH 4) was reduced by half while the
359 sugar content increased. The gamma temperature of *L. plantarum* indeed doubled from 25 °C
360 to 35-40 °C, thus explaining the more rapid fermentation while the activity of α-amylase,
361 predicted by the model was multiplied by 4. The bigger increase in α-amylase activity with
362 increasing temperature thus explained the increase in sugar content despite the acceleration of
363 fermentation. As far as the level of pre-cooking is concerned, the model showed (results not
364 shown) that the gelatinised part of starch was completely hydrolysed at the end of the
365 fermentation process (90% hydrolysis within 4-11 h) versus only 5-9% for native starch. Even
366 if the pre-cooking level was quite low (15 to 20%), the hydrolysis of the gelatinised starch
367 accounted for more than 60% of released sugars, which explains why the level of precooking
368 has a dramatic impact on the level of sugar in fermented *gowé*. Another consequence is that
369 increasing the level of malt will not have a significant impact on the sugar content of *gowé*. It
370 will only affect the hydrolysis of native starch (as gelatinised starch is already completely
371 hydrolysed with the present level of malt); for example, doubling the level of malt will only
372 increase the level of sugar in fermented *gowé* by 10-15%.

373 Two additional simulations were run with a fermentation temperature of 40 °C and an increased
374 level of pre-cooking (30 g pre-cooked at 85 °C). The amount of malt was not changed, but two
375 levels of LAB inoculum were tested. S2 simulation (Fig. 10) predicted a short fermentation
376 time (15 h), the highest sugar content (92 $g \cdot L^{-1}$), and a very low level of ethanol. In S1, a
377 significant proportion of sugar was indeed used by yeasts (due to the larger quantity of yeast
378 used for inoculation) to produce ethanol. S2 fermenting conditions thus appear to be optimum
379 to produce a safe (short fermentation period) *gowé* appreciated by consumers, with high sugar
380 content. In these conditions, the sugar content will be much higher than that measured in
381 uncontrolled *gowé* (25-50 $g \cdot L^{-1}$; Michodjehoun *et al.,* 2005, Vieira-Dalodé *et al.,* 2008). The
382 respective inoculation levels of LAB (10^6 CFU/g) and yeast (10^5 CFU/g) thus appear to be
383 optimum to allow rapid acidification, thereby contributing to the safety of the product while

leaving sufficient time for α-amylase to release sugars, thereby ensuring the final product is sufficiently sweet. However, it should be noted that the validation tests were performed in a practical way, i.e. by inoculating using dry active bacteria, which explains the relatively long lag phase (5 h) for LAB; this delay contributed to the release of sugars by α-amylase. Using a similar inoculation level but with active living LAB, Vieira-Dalodé *et al.* (2008) also showed that acidification time was shorter (7 h) but that the resulting *gowé* was less sweet and not better appreciated by consumers than the traditional *gowé*.

4. Conclusion

This work is the culmination of a step-by-step modelling approach using microbial growth models from predictive microbiology to construct an overall model of *gowé* fermentation by integrating a model of lactic fermentation by a selected *L. plantarum* strain, a model of the saccharification of starch by amylase activity of the malt, and the growth model of a selected *P. kluyveri* strain. Even if some discrepancies remain between the model and the experimental data, particularly the experimental over-growth of LAB, the model already clarifies the processes which occur in the product and makes it possible to extrapolate rapidly *in silico* various fermentation conditions. It thus enables prediction of the relative hydrolysis rate of native and gelatinised starches, while their respective individual assesment would be impossible. The model showed that increasing the proportion of malt is not an efficient way to increase the quantity of sugar in *gowé* whereas increasing of the level of pre-cooking and hence of starch gelatinisation is. We were thus able to propose optimum processing conditions for *gowé*.

More generally, the builing-block model developed in this study, able to describe the growth of yeast and lactic acid bacteria, starch hydrolysis, free sugar consumption and production of ethanol and lactic acid, could be applied to many other fermented foods and particularly to non-alcoholic but acid and sweet cereal based beverages.

Acknowledgments

This work was supported by the European funded project, AFTER, *Instituto Nacional de Gestão de Bolsas de Estudo* (INAGBE), *Ministério do Ensino Superior of the República de Angola,* French Agricultural Research Centre for International Development (CIRAD) and Embassy of France in Luanda (Angola). We thank Chr. Hansen Company (Prades-le-Lez,

France) for providing *Pichia kluyveri* strain (VINIFLORA® FROOTZEN®), and Ennolys (Soustons, France) for providing *Lactobacillus plantarum* (CNCM I-3069) strain.

References

Adinsi L., Vieira-Dalodé G., Akissoé N.H., Anihouvi V., Mestres C., Jacobs A., Dlamini N., Pallet D., Hounhouigan J.D. 2014. Processing and quality attributes of gowe: a malted and fermented cereal-based beverage from Benin. Food Chain, 4 (2): 171-183.

Adinsi L., Akissoé N.H., Dalodé-Vieira G., Anihouvi V., Fliedel G., Mestres C., Hounhouigan J.D.. 2015. Sensory evaluation and consumer acceptability of a beverage made from malted and fermented cereal: Case of gowe from Benin. Food Science and Nutrition, 3 (1): 1-9

Alegría, E., López, I., Ruiz, J.I., Sáenz, J., Fernández, E., Zarazaga, M., Dizy, M., Torres, C., and Ruiz-Larrea, F. 2004. High tolerance of wild *Lactobacillus plantarum* and *Oenococcus oeni* strains to lyophilisation and stress environmental conditions of acid pH and ethanol. FEMS Microbiol. Lett. *230*, 53–61

Arroyo-López, F.N., Querol, A., Bautista-Gallego, J., and Garrido-Fernández, A. 2008. Role of yeasts in table olive production. Int. J. Food Microbiol. *128*, 189–196.

Betts, G.D., Linton, P., and Betteridge, R.J. 1999. Food spoilage yeasts: effects of pH, NaCl and temperature on growth. Food Control *10*, 27–33.

Blandino, A., Al-Aseeri, M. E., Pandiella, S. S., Cantero, D., & Webb, C. 2003. Cereal based fermented foods and beverages. Food Research International, *36*(6), 527–543.

Charalampopoulos, D., Vázquez, J.A., and Pandiella, S.S. 2009. Modelling and validation of *Lactobacillus plantarum* fermentations in cereal-based media with different sugar concentrations and buffering capacities. Biochem. Eng. J. *44*, 96–105.

Cornu, M., Billoir, E., Bergis, H., Beaufort, A., and Zuliani, V. 2011. Modeling microbial competition in food: Application to the behavior of *Listeria monocytogenes* and lactic acid flora in pork meat products. Food Microbiol. *28*, 639–647.

Coroller, L., Guerrot, V., Huchet, V., Lemarc, Y., Mafart, P., Sohier, D., and Thuault, D. 2005. Modelling the influence of single acid and mixture on bacterial growth. Int. J. Food Microbiol. *100*, 167–178.

De Levie, R. 2001. How to use Excel in analytical chemistry and in general scientific data analysis (Cambridge; New York: Cambridge University Press).

Delgenes, J.P., Moletta, R., and Navarro, J.M. 1988. The ethanol tolerance of *Pichia stipitis* Y 7124 grown on a D-xylose, D-glucose and L-arabinose mixture. J. Ferment. Technol. *66*, 417–422.

Du Preez, J.C. 1994. Process parameters and environmental factors affecting d-xylose fermentation by yeasts. Enzyme Microb. Technol. *16*, 944–956.

Gobbetti, M., Corsetti, A., and Rossi, J. 1994. The sourdough microflora. Interactions between lactic acid bacteria and yeasts: metabolism of carbohydrates. Appl. Microbiol. Biotechnol. *41*, 456–460.

Lonvaud, A., Renouf, V., and Strehaiano, P. 2010. Microbiologie du vin : bases fondamentales et applications. Ed Tecdoc Lavoisier, Cachan, France.

Mestres C., Nguyen T.C., Adinsi L., Hounhouigan J.D., Fliedel G., Loiseau G. 2015. The interaction between starch hydrolysis and acidification kinetic determines the quality of a malted and fermented sorghum beverage. J. Cereal Sci., 63: 8-13.

Mestres, C., Munanga, B.J.C., Loiseau, G., Matignon, B., Grabulos, J., and Achir, N. 2017. Modeling cereal starch hydrolysis during simultaneous saccharification and lactic acid fermentation; case of a sorghum-based fermented beverage, gowé. Food Res. Int. *100*, 102-111.

Michodjehoun-Mestres L., Hounhouigan J.D., Dossou J., Mestres C. 2005. Physical, chemical and microbiological changes during natural fermentation of "gowé", a sprouted or non sprouted sorghum beverage form West-Africa. African Journal of Biotechnology, 4 (6): 487-496.

Munanga, B., Loiseau, G., Grabulos, J., and Mestres, C. 2016. Modeling lactic fermentation of Gowé using *Lactobacillus* starter culture. Microorganisms *4*, 44.

Mussatto, S.I., Machado, E.M., Carneiro, L.M., and Teixeira, J.A. 2012. Sugars metabolism and ethanol production by different yeast strains from coffee industry wastes hydrolysates. Appl. Energy *92*, 763–768.

Nout MJR, Rombouts FM, Havelaar A 1989. Effect of accelerated natural lactic fermentation of infant food ingredients on some pathogenic micoorganisms. Int. J. Food Microbiol. 8: 351 – 361.

Nout MJR. 1991. Ecology of accelerated natural lactic fermentation of sorghum based infant food formulas. Int. J. Food Microbiol. 12: 217-224.

Praphailong, W., and Fleet, G.H. 1997. The effect of pH, sodium chloride, sucrose, sorbate and benzoate on the growth of food spoilage yeasts. Food Microbiol. *14*, 459–468.

Rosso, L. 1995. Modélisation et microbiologie prévisionnelle : Élaboration d'un nouvel outil pour l'Agro-alimentaire. Université Claude Bernard-Lyon I.

Rosso, L., Lobry, J.R., Bajard, S., and Flandrois, J.P. 1995. Convenient model to describe the combined effects of Temperature and pH on microbial growth. Appl. Environ. Microbiol. *61*, 610–616.

Rouwenhorst, R.J., Visser, L.E., Van Der Baan, A.A., Scheffers, W.A., and Van Dijken, J.P. 1988. Production, distribution, and kinetic properties of inulinase in continuous cultures of *Kluyveromyces marxianus* CBS 6556. Appl. Environ. Microbiol. *54*, 1131–1137.

Sharma, V. and Mishra, H.N. 2014. Unstructured kinetic modeling of growth and lactic acid production by *Lactobacillus plantarum* NCDC 414 during fermentation of vegetable juices. LWT - Food Sci. Technol. *59*, 1123–1128.

489 Song, H., Jang, S.H., Park, J.M., and Lee, S.Y. 2008. Modeling of batch fermentation kinetics
490 for succinic acid production by *Mannheimia succiniciproducens*. Biochem. Eng. J. *40*, 107–
491 115.

492 Thomas, K.C., Hynes, S.H., and Ingledew, W.M. 2002. Influence of medium buffering capacity
493 on inhibition of *Saccharomyces cerevisiae* growth by acetic and lactic acids. Appl. Environ.
494 Microbiol. *68*, 1616–1623.

495 Van Impe, J., Poschet, F., Geeraerd, A., and Vereecken, K. 2005. Towards a novel class of
496 predictive microbial growth models. Int. J. Food Microbiol. *100*, 97–105.

497 Vieira-Dalode, G. Jespersen, L.Hounhouligan, J.Moller, P. L.Nago, C. M. and Jakobsen, M.,
498 2007. Lactic acid bacteria and yeasts associated with gowe production from sorghum in Benin.
499 J. Applied Microbiol. 103(2): 342-349.

500 Vieira-Dalode, G., Madode, Y. E.Hounhouigan, J.Jespersen, L. and Jakobsen, M. 2008. Use of
501 starter cultures of lactic acid bacteria and yeasts as inoculum enrichment for the production of
502 gowe, a sour beverage from Benin."African Journal of Microbiology Research 2(7): 179-186.

503 Viljoen, B.C. 2006. Yeast ecological interactions. Yeast–yeast, yeast–bacteria, yeast–fungi
504 interactions and yeasts as biocontrol agents. In yeasts in food and beverages. Berlin Heidelberg
505 New York: Springer-Verlag, pp. 83–110.

506 Zwietering, M.H., Wijtzes, T., Rombouts, F.M., and Van't Riet, K. 1993. A decision support
507 system for prediction of microbial spoilage in foods. J. Ind. Microbiol. *12*, 324–329.

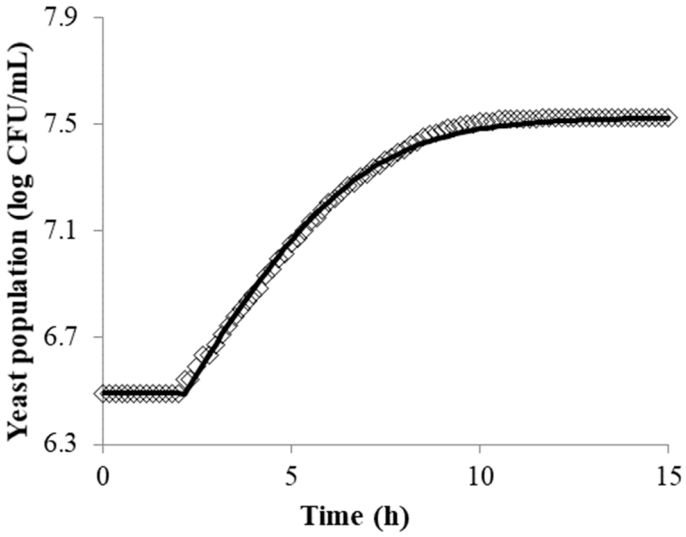

Figure 1: Growth curve of *Pichia kluyveri* in malt extract broth at 30 °C and pH 6.5. Experimental data (◊) and predicted data (continuous line)

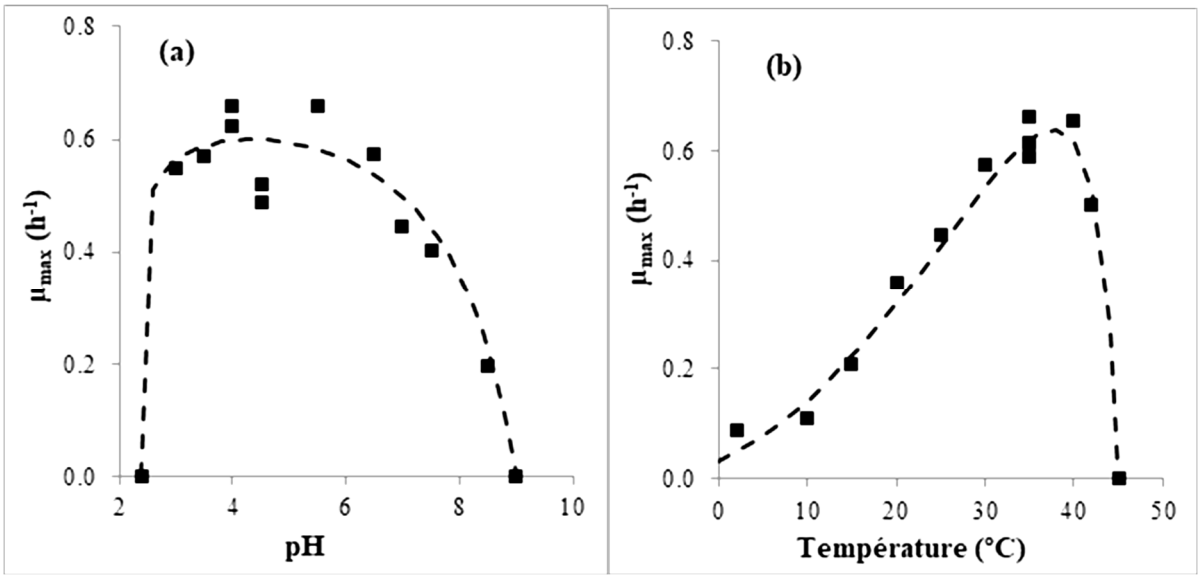

Figure 2: Effect of environment factors on growth rate of *Pichia kluyveri*. pH effect (**a**) and temperature effect (**b**). Experimental data (symbol) and predicted data (dotted line).

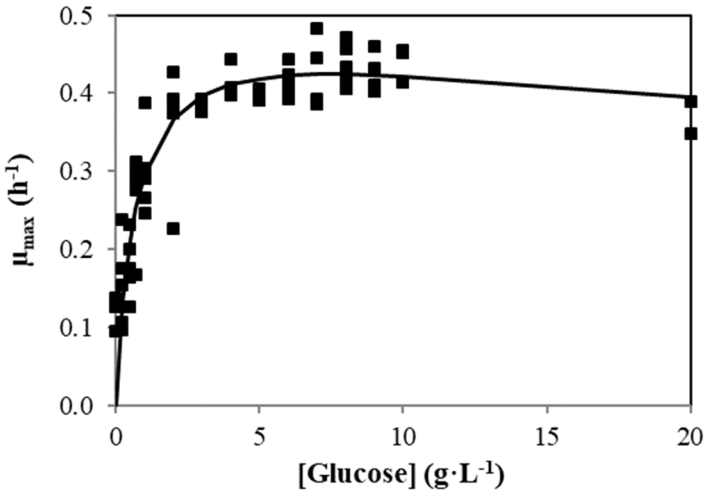

Figure 3: Effect of glucose concentration on *Pichia kluyveri* growth rate. Experimental data (symbol) and predicted data (dotted line).

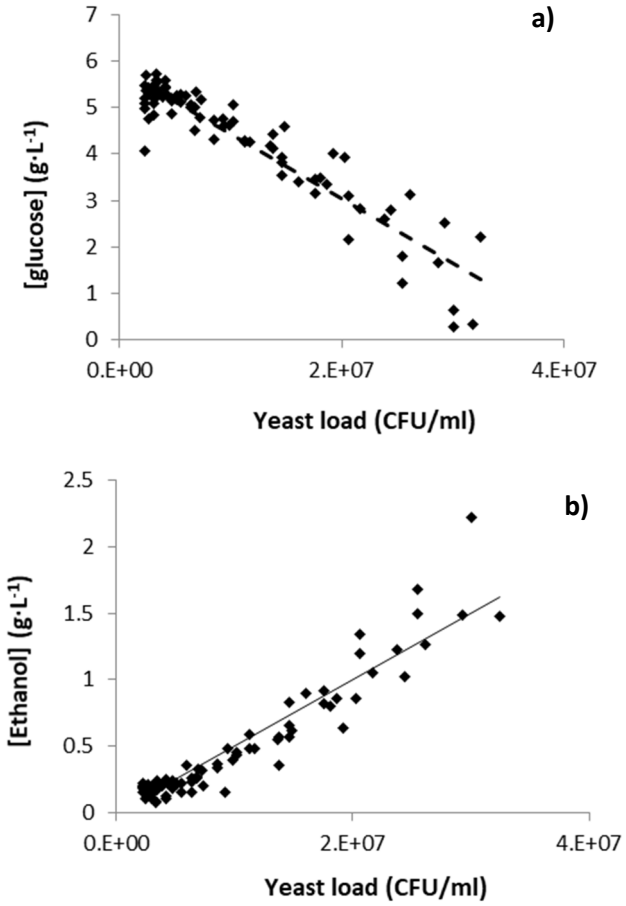

Figure 4: Regression between glucose concentration (a), ethanol concentration (b) and microbial load for *Pichia kluyveri* at different growth conditions of temperature and pH. Experimental data (symbol) and predicted data (dotted line).

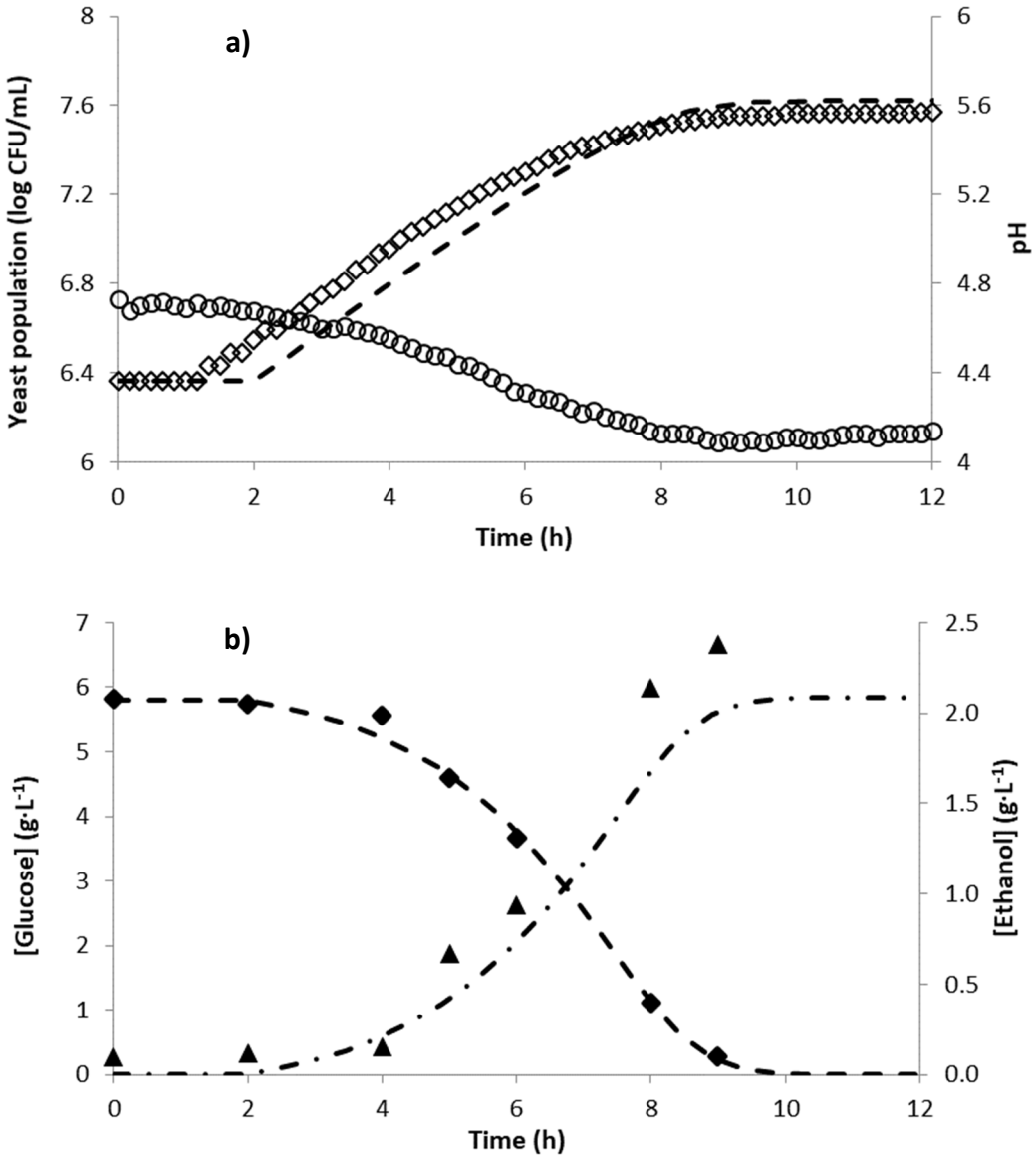

Figure 5: Changes in the yeast population (◊, - - -) and pH (○) (a), glucose (♦, - - -) and ethanol (▲, -.-.-) concentrations (b) for the validation test performed at 30°C in YPG broth inoculated with *Pichia kluyveri*. Experimental data (symbol) and predicted data (dashed lines)

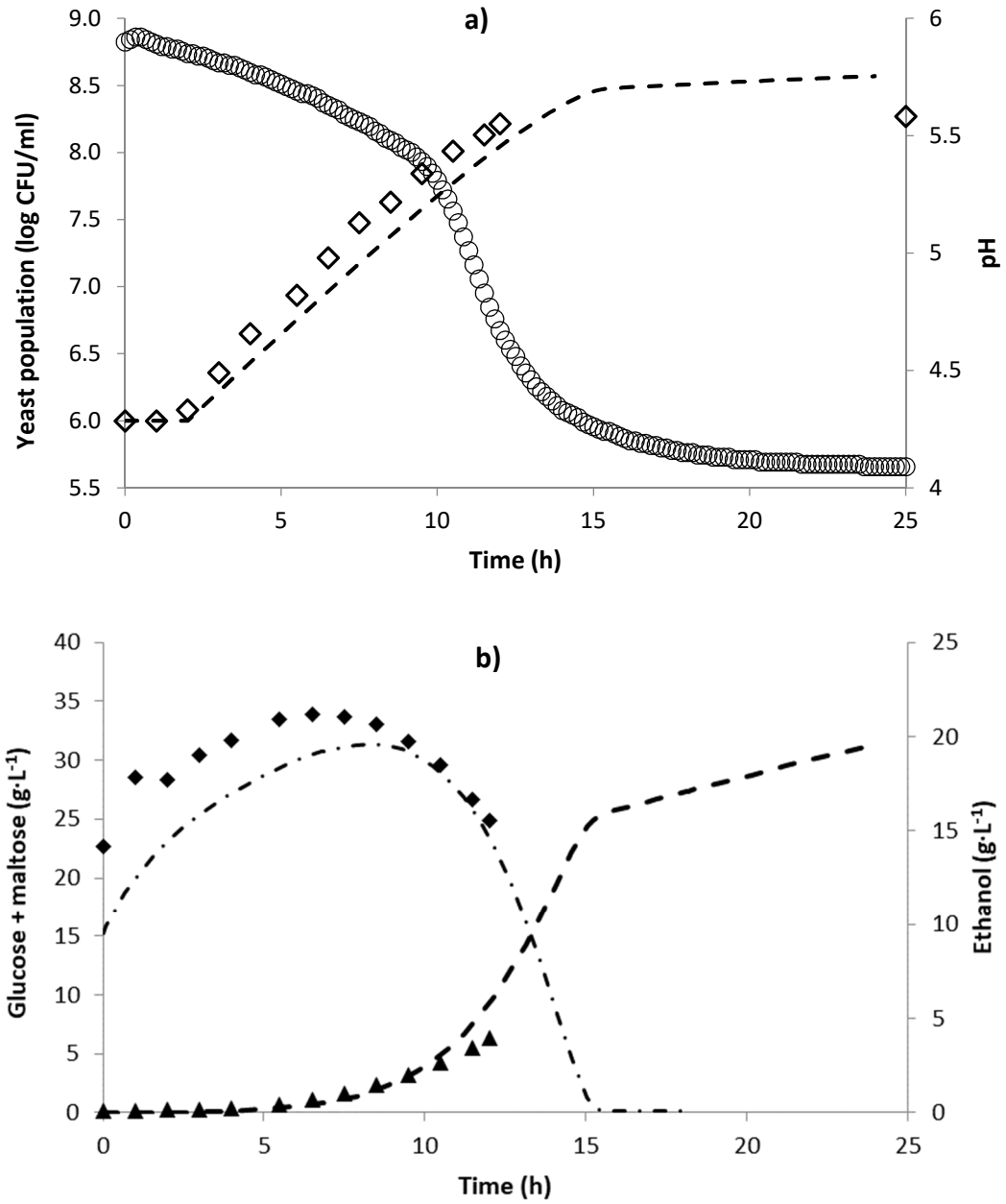

Figure 6: Changes in yeast population (◊, - - -) and in pH (○) (a), glucose and maltose (♦, -.-.-) and ethanol (▲, - - -) concentrations (b) for the validation test for the fermentation of gowé at 30°C inoculated with *Pichia kluyveri*. Experimental data (symbol) and predicted data (dashed line)

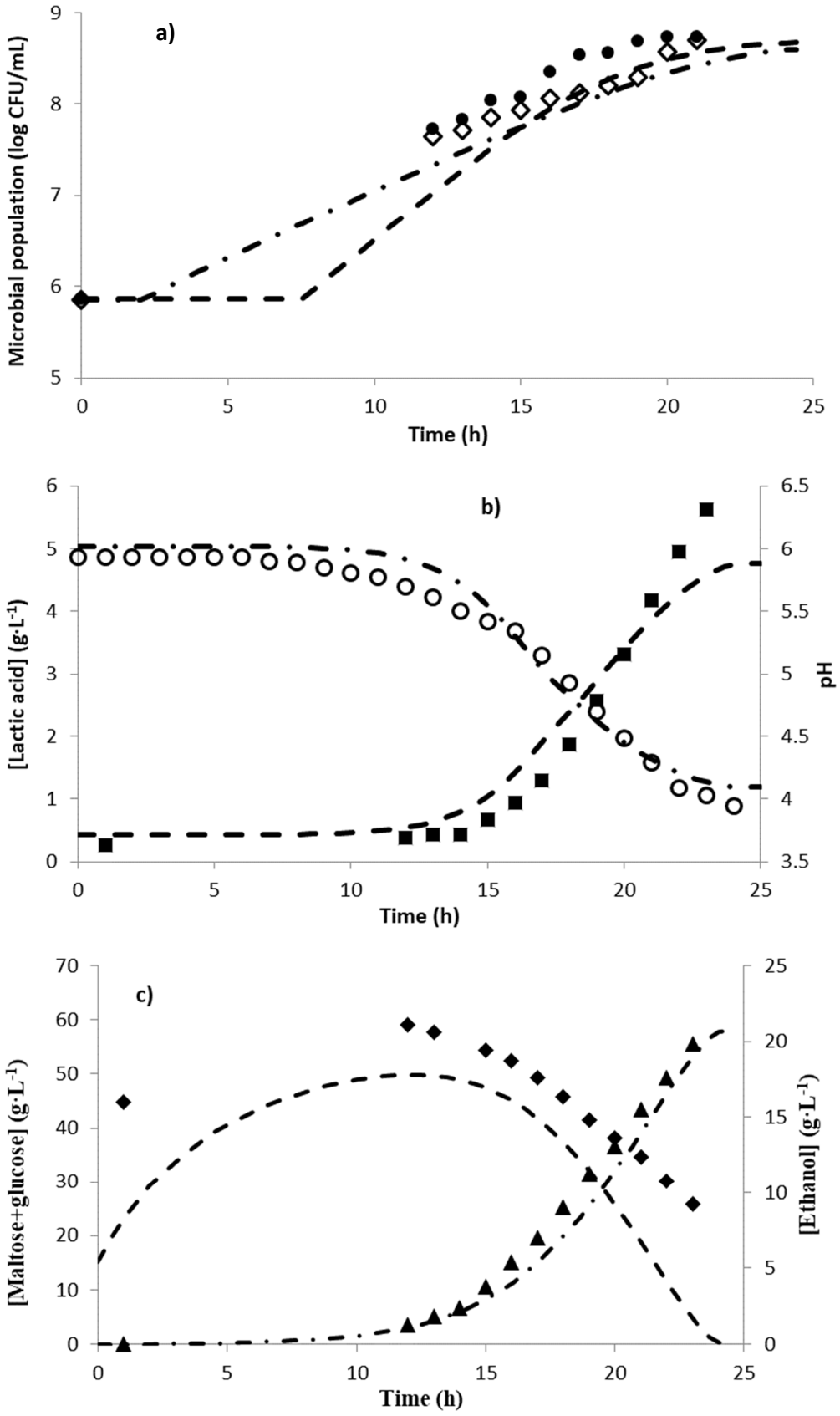

Figure 7: **(a)** Changes in the population of *Lactobacillus plantarum* (●, - - -) and *Pichia kluyveri* (◊, -.-.-)**; (b)** in pH (○, -.-.-) and lactic acid concentration (■, - - -)**; (c)** maltose + glucose (♦, - - -) and ethanol (▲, -.-.-) concentrations for validation test V1. Experimental data (symbol), predicted values (dashed lines)

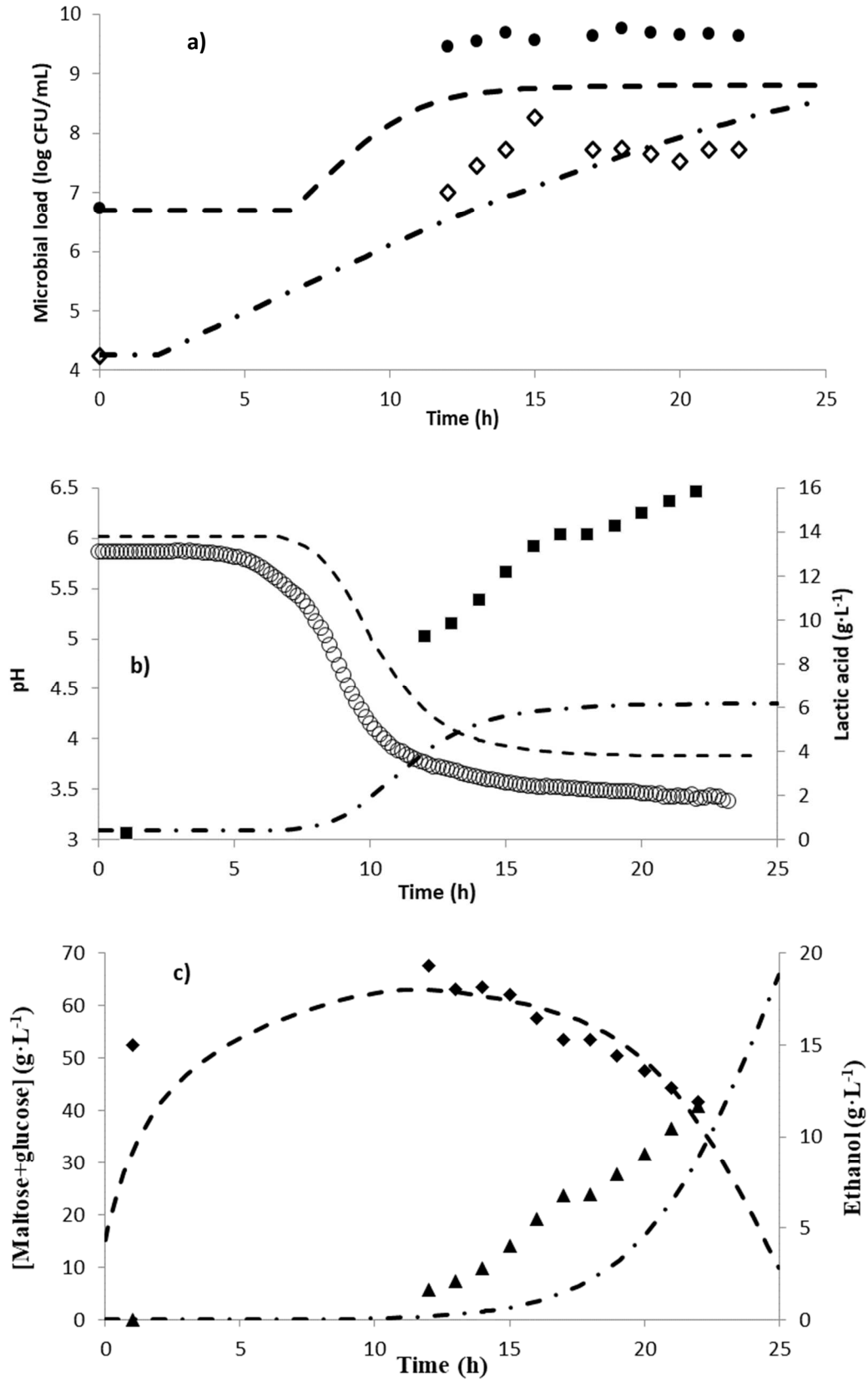

Figure 8: **(a)** Changes in the population of *Lactobacillus plantarum* (●, - - -) and *Pichia kluyveri* (◊, -.-.-)**; (b)** in pH (○, -.-.-) and lactic acid concentration (■, - - -)**; (c)** maltose + glucose (♦, - - -) and ethanol (▲, -.-.-) concentrations for validation test V2. Experimental data (symbol) and predicted values (dashed lines)

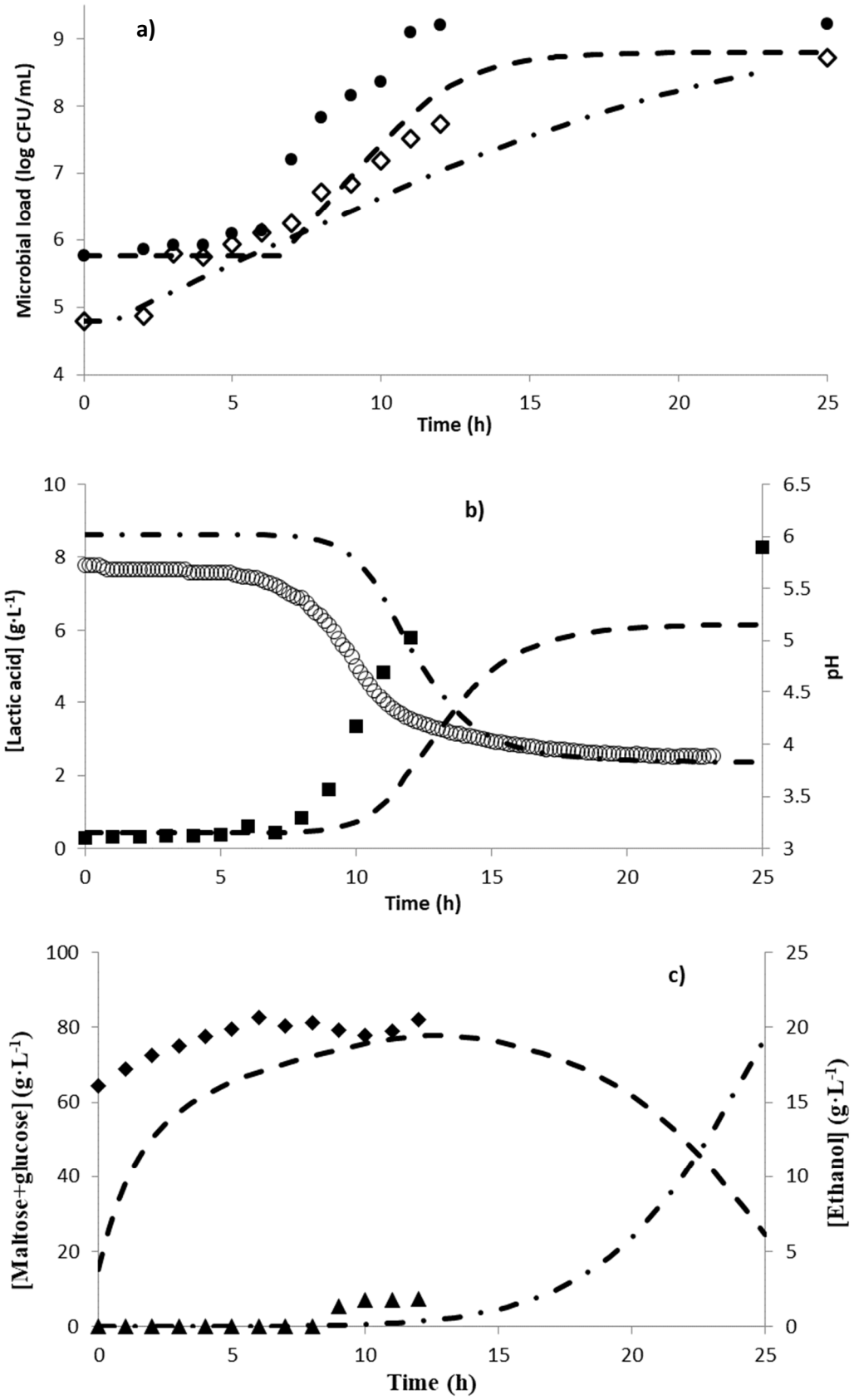

Figure 9: **(a)** Changes in the population of *Lactobacillus plantarum* (●,- - -) and *Pichia kluyveri* (◊, -.-.-)**; (b)** in pH (○,-.-.-) and lactic acid concentration (■, - - -)**; (c)** maltose + glucose (♦, - - -) and ethanol (▲, -.-.-) concentrations for validation test V3. Experimental data (symbol) and predicted values (dashed lines)

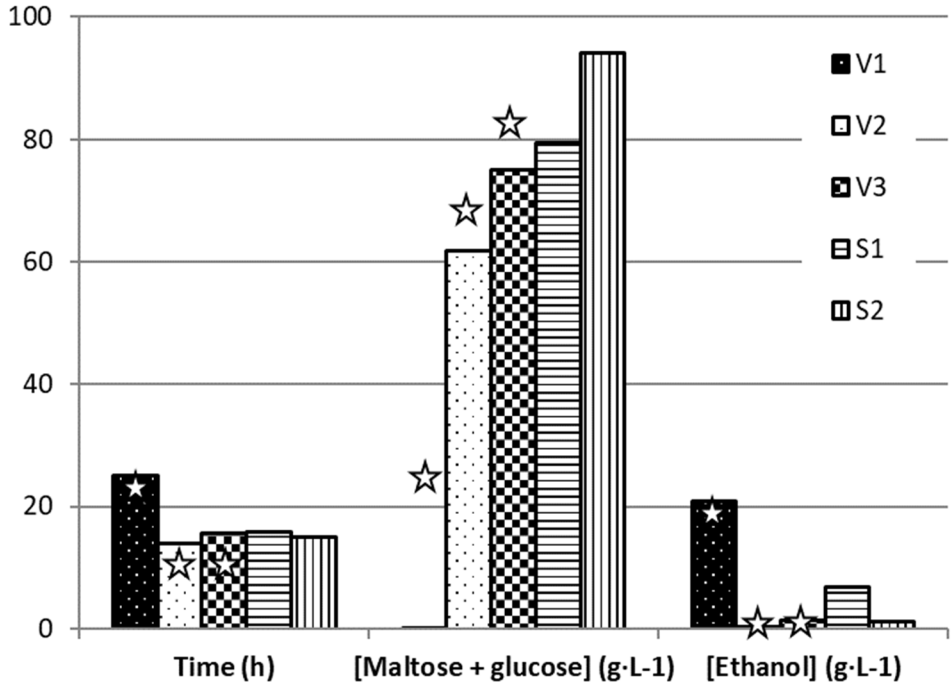

Figure 10: Predicted values of time of fermentation and of final concentrations of maltose + glucose and of lactic acid for the validation (V1-V3) and simulation tests (S1, S2). Observed values for the validation tests are positioned in the form of stars