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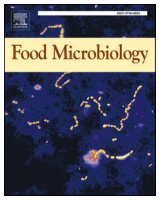

Investigating the growth kinetics in sourdough microbial associations

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ABSTRACT

In this study we investigated the effect of the single strain in stabilization of type I sourdough microbial associations by crossing six different *Fructilactobacillus sanfranciscensis* with five *Kazachstania humilis* strains. Furthermore, we compared three predictive models, Zwitwering based on Gompertz's equation, Baranyi and Roberts' function and Schiraldi's function to evaluate which one best fitted the experimental data in determining the behaviour of co-cultivated microorganisms. Specific growth rates (μ_m) and lag time (λ) values for each mixed population were assessed. Results showed that the different *F. sanfranciscensis* strains significantly steer the growth kinetics within the pair and affect the ratio bacterial/yeast cells, as data analysis confirmed, whereas *K. humilis* accommodates to the bacterial strain. To compare the growth models, Root Mean Square (RMS) values were calculated for each predicted curve by implementing an algorithm based on an iterative process to minimize the deviation among observed and calculated data. Schiraldi's function performed better than the others, revealing, on average, the smallest RMS values and providing the best fitting for over 70% of co-cultivation experiments. Models prove to be consistent in predicting growth kinetics of microbial consortia too.

1. Introduction

The dominance of the species *Fructilactobacillus sanfranciscensis*, formerly *Lactobacillus sanfranciscensis*, and *Kazachstania humilis*, formerly *Candida milleri*, in type I sourdough is a fascinating phenomenon of microbial mutualism that has been reviewed in several works (Corsetti and Settanni, 2007; De Vuyst et al., 2014; Gänzle and Zheng, 2019; Gobbetti et al., 2016; Lattanzi et al., 2013; Picozzi et al., 2006). The formation of this spontaneous consortium in cereal doughs and its stability along the time are explained by non-competition for carbon sources, metabolites exchanges, resistance to acidity, likeness in behaviour toward temperature and redox potential and common ecological origin (Boiocchi et al., 2017; Brandt et al., 2004; De Vuyst et al., 2009; De Vuyst and Neysens, 2005; Ehrmann and Vogel, 1998; Gobbetti, 1998; Venturi et al., 2012; Vogel et al., 1999). Furthermore, environment and technological parameters such as temperature, dough yield, type of flour and fermentation time, clearly affect the settlement of specific microbial communities, as the traditional widespread back-slopping technique demonstrates (Gobbetti et al., 2005; Minervini et al., 2012a, 2012b; Siragusa et al., 2009; Vrancken et al., 2011; Wick et al., 2003). The conservative bread-making practices, rigorously made to keep on the type I sourdough process, force the evolution of the microbial populations towards very few dominant strains (Meroth et al., 2003; Picozzi et al., 2010; Van der Meulen et al., 2007; Viiard et al., 2012), often one yeast and one bacterial strains (De Vuyst et al., 2014; Carbonetto et al., 2018), which maintain a constant equilibrium among them. What remains to be elucidated is if a single *F. sanfranciscensis* strain will develop an identical relationship with different *K. humilis* strains and *vice versa*. Besides, when the same environmental conditions are given, we do not know if and how the numerical ratio among bacterial and yeast cells becomes stable.

Primarily, this evaluation may be interesting to speculate the interactions between the two species. It is currently arguable if consortium stability is determined by similarity in growth rates or whether one partner steers the other. Secondly, it can be useful in industrial microbiology for optimizing the biomass production with mixed starter cultures when microorganisms are co-cultivated.

The analysis of experimental results from growth tests in which each

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Table 1
Microbial strains used in this work. Most of them are isolate in Italy. Yeast strain UMY98 corresponds to CBS5658^T.

Species	Strain	Type of product	Place of isolation	Time of isolation
K. humilis	UMY1	Panettone sourdough	Sondrio	2006
K. humilis	UMY9	bread sourdough	Modena	2003
K. humilis	UMY10	bread sourdough	Modena	2003
K. humilis	UMY12	Colomba sourdough	Milano	2012
K. humilis	UMY98	Bantu beer	South Africa	1979
F. sanfranciscensis	UMB9	Panettone sourdough	Pavia	1994
F. sanfranciscensis	UMB21	Brioche sourdough	Verona	2001
F. sanfranciscensis	UMB36	Bread sourdough	Verona	2001
F. sanfranciscensis	UMB37	Pugliese sourdough	Verona	1998
F. sanfranciscensis	UMB42	Bread sourdough	Campobasso	1998
F. sanfranciscensis	UMB49	Bread sourdough	Catania	2007

strain is inoculated in single or coupled with another (one bacterium *vs* one yeast) can lead to understand what happens through the comparison of values obtained in kinetics parameters and eventually to predict what will occur.

So far, modelling of microbial growth has been mainly directed to an empirical approach based on sigmoidal functions that well fit data of cell concentration *vs* time. The most used equations are that of Zwitwering et al. (1990) based on Gompertz's model and that of Baranyi and Roberts (1994), termed as primary functions. They assume binary fission as cell multiplication, planktonic conditions, and unlimited nutrient substrates. Three parameters with biological meanings are identified in Zwitwering's function: the maximum specific growth rate (μ_m), which is defined as the tangent in the inflection point of the growth curve; the lag time (λ), which is defined as the x-axis intercept of this tangent; and the asymptote of the curve at infinite time, which is the maximal value reached by the cell concentration. Baranyi and Roberts (1994) introduced an additional variable concerning the physiological state of the cells. The values of these parameters can change because of variation, within specific biological limits, of some environmental factors, such as temperature, pH, activity water, redox potential, and they are counted through the application of other mathematical equations, termed secondary functions.

Starting from the same presumptions, Schiraldi (2017) recently proposed a two-parameter model of microbial growth that considers the non-synchronous progress of cell generations. In particular, it assumes that the generation time of each individual cell line changes during time with some constraints supported by phenomenological evidence, such as zeroing of growth rate at the beginning and at the end of the process or presence of non-generating cells. Albeit no biological parameter appears in the equation, the experimental data retrieved in the literature and those calculated by the function showed a very good agreement, supporting the hypothesis that growth kinetics follow auto-regulating controls not depending on the type of microorganism itself (Schiraldi, 2017). This assumption could unfasten the application of the model from the study of a single strain to that of mixed populations, even for cells that do not duplicate but multiply by budding, such as yeasts.

Thus, the objectives of this investigation are two: to understand the influence of a single strain in building of microbial consortium that dominates in type I sourdough; to compare the results generated by three growth models with the purpose of verifying which is more performant in predicting the whole cellular development of communities composed by two different populations.

2. Materials and methods

2.1. Microorganisms and media

Six *F. sanfranciscensis* strains previously genotyped by Picozzi et al. (2010) and five *K. humilis* strains, previously genotyped by Vigentini et al. (2014), have been selected to infer the effect of the single microorganism on the growth kinetics of the microbial consortium. All strains, but *K. humilis* CBS5658^T, originated from Italian type I sourdough samples and they varied in genotype, place and time of isolation (Table 1). LAB and yeast cultures have been prepared, maintained and counted according to Picozzi et al. (2005) and Foschino et al. (2004), respectively. The formulation of modified SanFrancisco Medium for the cultivation of *F. sanfranciscensis* strains has been: tryptone10 g/L, meat extract 2 g/L, yeast extract 7 g/L, glucose 7 g/L, fructose 7 g/L, maltose 7 g/L, sodium acetate $3H_2O$ 5 g/L, citric acid (diammonium salt) 5 g/L, KH_2PO_4 $3H_20$ 2.5 g/L, sodium gluconate 2 g/L, Tween 80 1 g/L, cysteine-HCl 0.5 g/L, $MgSO_4$ $7H_2O$ 0.2 g/L, $MnSO_4$ $4H_20$ 0.05 g/L, $FeSO_4$ $7H_2O$ 0.01 g/L, pH = 5.4; agar 15 g/L if needed. The liquid part has consisted of demineralised water 850 mL and 150 mL Fresh Yeast Extract, so that the medium remained clear (Picozzi et al., 2005). The formulation of YEPD medium for the cultivation of *K. humilis* strains has been: glucose 20 g/L, peptone 20 g/L, yeast extract 10 g/L, pH = 6.2; agar 15 g/L if needed.

2.2. Co-cultivation experiments

Preliminary tests have been carried out to set up the composition of a liquid medium suitable for the growth of microorganisms in co-cultivation and to maximize the biomass production in view of an industrial implementation. The chosen broth, named M40, had the following composition: maltose 40 g/L, peptone 15 g/L, yeast extract 10 g/L, fructose 5 g/L, Tween80 1 mL/L, $MgSO_4$ 0.2 g/L, $FeSO_4$ $7H_2O$ 0.01 g/L, $MnSO_4$ 0.005 g/L, pH 6.0.

Each bacterial strain has been grown together with each yeast strain in co-cultivation experiments. Single *F. sanfranciscensis* strains have been cultivated as controls; conversely, single *K. humilis* strains were not used as controls since they have been not able to well grow alone in the tested medium, being maltose-negative yeasts. Calibration curves between plate counts and optical density (OD) values at 600_{nm} (Jenway, UV–visible spectrophotometer, model 7315, Bibby Scientific, Stone, England) of fresh cultures have been constructed to arrange a desired starting inoculum (supplementary materials Fig. 1S). Standardized cell concentrations have been prepared by growing overnight cultures in modified SanFrancisco medium (Picozzi et al., 2005) for *F. sanfranciscensis* strains and in YEPD broth, under agitation at 200 rpm, for *K. humilis* strains (Foschino et al., 2004). Fresh cells have been collected and washed by centrifugation at 4000*g* at 4 °C for 15 min (Hettich centrifuge, Rotina 380, Tuttlingen, Germany) and resuspended in the respective cultural broths. At the beginning of the experiment, bacterial cells have been approximately set to 2.4×10^8 CFU/mL and yeasts to 4.8×10^6 CFU/mL, in order to get an initial ratio of 50:1. This choice was made on the basis of previous experiences and calculation by extrapolating the median values of LAB and yeast counts reported by Minervini et al.(2012a) and Scheirlinck et al. (2007). The growth tests have been carried out at 25 °C ± 1 °C in 96-wells polystyrene microplates with 200 μL volumes of the above-mentioned broth for each well. The OD at 600_{nm} of the cultures have been monitored by a Plate Reader (Infinite 200pro, Tecan Group, Zurich, Switzerland) in 9 different points of the well every 20 min for 24 h after 10-s shaking by an amplitude of 1 mm.

After 24 h of incubation, 4 μL of microbial suspension have been taken from each well to be inoculated into 200 μL of fresh medium placed in another well prepared in new microplates. This step has been

repeated twice in order to investigate the stability of the ratio bacterial/yeast cells during time.

2.3. Estimation of the ratio bacterial/yeast cells

At 24 h of incubation for each refreshment, 10 μL cell suspensions have been sampled after thoroughly mixing, and observed by an optical microscope (Axio Lab. A1 model, Zeiss, Oberkochen, Germany) at 400 X under phase contrast.

Images of the microscopical field have been collected through a digital camera (OM-D, Olympus, Shinjuku, Japan) and elaborated by a self-built function developed in MATLAB® environment (R2019a, MathWorks, Natick, U.S.A.) able to sort and separately count bacterial and yeast cells. The image analysis procedure has allowed to obtain a binary image from the red channel filled with zero values for pixels recognised as background and one values for pixels possibly attributed to microorganisms. After the cleaning procedure of the image the differentiation between LAB and yeast has been performed based on the object area in the obtained binary image. Object with area in the range 300–500 pixels have been attributed to yeast cells, whereas object composed by 80–300 pixels have been recognised as bacterial ones; these thresholds have been previously defined by observing pure cultures of the strains under the same conditions. So that the ratio bacterial/yeast cells has been expressed as the proportion between small (area <300 pixels) and big (area >300 pixels) objects.

2.4. Shaping the growth of a microbial consortium

A purely empirical approach has been pursued based on Schiraldi's deduction (Schiraldi, 2017), namely that a growth model can be untied from processes of cell development implying a duplication mechanism, since many coexisting generation lines lead to a convolution of the system.

By plotting the optical density values of the microbial consortia *vs* time, the observed patterns always assumed sigmoidal forms. The essential characteristics of the sigmoidal curves have been calculated with the procedure described below, allowing to determine values of maximum growth rate (μ_m) and lag time (λ), without *a priori* growth model definition.

Firstly, only values within the minimum and maximum of the dataset have been considered, thus excluding the early settling phase and the late mortality phase, not explained by the models. A mathematical transformation of OD values into logarithms was performed to guarantee data normal distribution needed for the implementation of the growth models.

Secondly, a preliminary identification of the inflection point (t_{f0}) has been carried out by finding the part of the curve with maximum slope.

Thirdly, a fitting has been performed by applying a third-degree polynomial function with MATLAB® environment ("polyfit" function) to the values for each growth test, centred in t_{f0}:

$$a + b(t - t_{f0}) + c(t - t_{f0})^2 + d(t - t_{f0})^3$$

From the coefficients of the polynomial function, the values of the log(N/N$_0$) at the inflection point (Y_f) and inflection time ($t_f = t_{f0} - \frac{c}{3d}$) have been calculated.

Finally, by analogy with representation of pure culture growth, values of μ_m and λ have been obtained for each growth test.

$$\mu m = b - \frac{c^2}{3d}$$

$$\lambda = t_f - \frac{Y_f}{\mu}$$

2.5. Comparison of growth models

Since each growth model considers a different number of parameters, it has been deemed appropriate to bring them to a same number to objectively compare the fitness of the functions to the experimental data in the same way.

The investigated growth models were:

The Gompertz's function modified by Zwietering et al. (1990),

$$Y = \ln\left(\frac{N}{N_0}\right) = A \exp\left\{ - \exp\left[\frac{\mu e}{A}(\lambda - t) + 1\right]\right\}$$

the Baranyi and Roberts' function (1994),

$$Y = \ln\left(\frac{N}{N_0}\right) = \mu A(t) - \ln\left(1 + \frac{\exp(\mu A(t)) - 1}{\exp(Y_{max})}\right)$$

where,

$$A(t) = t + \frac{1}{\mu}\ln\{\exp(-\mu t) + \exp(-\mu\lambda) - \exp[-\mu(t + \lambda)]\}$$

and the Schiraldi's function (2017).

$$Y = \ln\left(\frac{N}{N_0}\right) = \ln(2)\frac{(t - t_0)^2}{a + b(t - t_0)^2}.$$

All above-mentioned mathematical expressions have been implemented into MATLAB® environment.

For Baranyi and Roberts' function, the curvature parameter "m" has been assumed equal to 1, as suggested by the same authors and Buchanan et al. (1997). Moreover, since the actual environmental conditions may be presumed as constant, the parameter "ν" has been set equal to μ_m.

For Schiraldi's model, the two parameters "a" and "b" were considered as such; a time shift has been inserted to take account of the latency phase, according to the author's recommendation (Schiraldi, 2017).

In this way, all growth models presented three free parameters, which have been found with the "nlinfit" function of MATLAB® environment; the coefficients have been assessed using iterative Least Squares estimation. The goodness of the fit for each growth test has been evaluated by determining Root Mean Square (RMS) value obtained as:

$$RMS = \sqrt{\frac{\sum_{i=1}^{N}\left(Y_{i\ exp} - Y_{i\ fit}\right)^2}{N}}$$

where N is the number of experimental points on the curve.

2.6. Statistical analysis

The effect of some factors (bacterial/yeast strain, refreshment number) on μ_m and λ have been investigated by carrying out ANOVA according to the general linear model. IBM SPSS statistics V26 software was used for data processing and graphs drawing. When the effect of a factor was significant ($p < 0.001$), discrimination was based on Tukey's HSD multiple comparisons test, at 95% confidence level.

3. Results

3.1. The F. sanfranciscensis strain steers the growth kinetics of the microbial association

180 co-cultivation growth tests were carried out by crossing each *F. sanfranciscensis* strains (number 6) for each *K. humilis* strains (5), for consecutive refreshments (3) and all twice replicated, plus 36 growth tests, in the same conditions, with each bacterial strain inoculated alone (controls). The initial ratio bacterial/yeast cells in co-cultivation experiments was set at about 50, approximately matching to OD_{600nm}

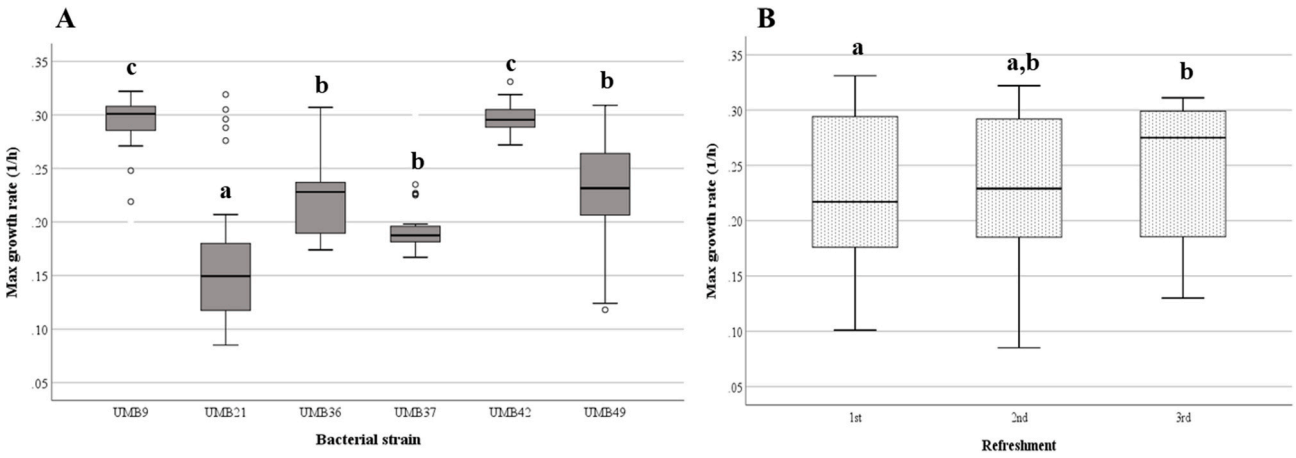

Fig. 1. Effect of bacterial strain (panel A) or refreshment (panel B) on maximum growth rate in co-cultivation experiments with different strains of *F. sanfranciscensis* and *K. humilis*. Box-and-whisker plots with different superscripts letters are significantly different ($p < 0.05$). Tukey's HSD test was performed with interval confidence at 95%.

Table 2
ANOVA results of maximum growth rate values observed in co-cultivation experiments.

Source	Sum Sq	d.f.	Mean Sq	F	Prob > F	
bacterial strain	0.36532	5	0.07306	53.28	0	***
yeast strain	0.00734	4	0.00183	1.34	0.2598	n. s.
refreshment	0.03322	2	0.01661	12.11	0	***
bacterial strain x yeast strain	0.02248	20	0.00112	0.82	0.6863	n. s.
bacterial strain x refreshment	0.05078	10	0.00508	3.7	0.0002	**
yeast strain x refreshment	0.03161	8	0.00395	2.88	0.0056	*
error	0.17142	125	0.00137			
total	0.68136	174				

*** means $p < 0.001$, **$p < 0.01$, *$p < 0.05$, n.s. = not significant difference.

values of 0.22 for bacteria and 0.09 for yeasts, in accordance with the respective calibration curves. The same starting cell density was provided for control tests with *F. sanfranciscensis* strains.

The assessment of μ_m and λ values for the mixed populations formed by each pair of strains and relevant control was made by applying the protocol described in 2.4 paragraph. Data from six curves were excluded from subsequent elaboration because the tests were contaminated or inoperable.

The ANOVA of results allowed to highlight significant differences ($p < 0.001$) between the mean values of μ_m shown by co-cultures with

Table 3
ANOVA results of duration time of lag phase observed in co-cultivation experiments.

Source	Sum Sq	d.f.	Mean Sq	F	Prob > F	
bacterial strain	70.274	5	14.055	18.24	0	***
yeast strain	3.427	4	0.857	1.11	0.3545	n. s.
refreshment	384.152	2	192.076	249.25	0	***
bacterial strain x yeast strain	11.096	20	0.555	0.72	0.7985	n. s.
bacterial strain x refreshment	89.154	10	8.915	11.57	0	***
yeast strain x refreshment	2.256	8	0.282	0.37	0.9364	n. s.
error	87.08	113	0.771			
total	675.895	174				

*** means $p < 0.001$, **$p < 0.01$, *$p < 0.05$, n.s. = not significant difference.

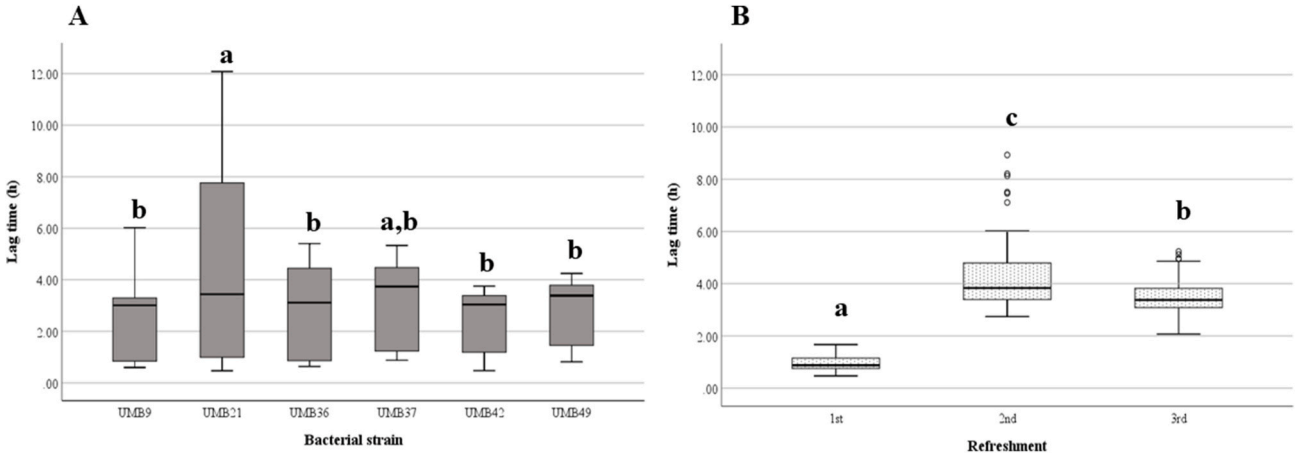

Fig. 2. Effect of bacterial strain (panel A) or refreshment (panel B) on duration time of lag phase in co-cultivation experiments with different strains of *F. sanfranciscensis* and *K. humilis*. Box-and-whisker plots with different superscripts letters are significantly different ($p < 0.05$). Tukey's HSD test was performed with interval confidence at 95%.

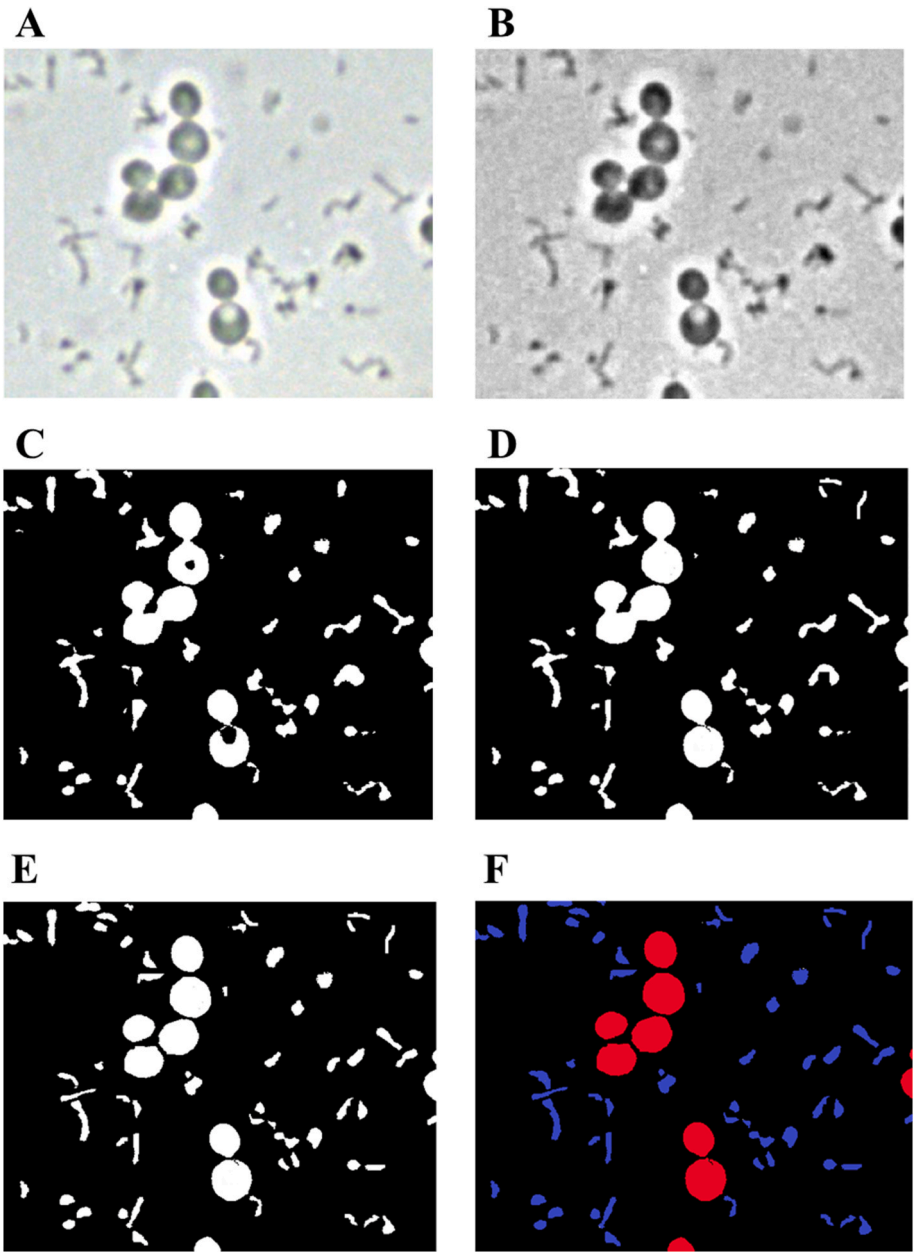

Fig. 3. Image analysis procedure on one portion of the image acquired for *K. humilis* UMY1strain combined with *F. sanfranciscensis* UMB42 strain: A) original image, B) red channel, C) binary image from the red channel, D) binary image after object erosion and dilatation, E) binary image after the improvement of cell edges, F) classification of yeast (red) and bacteria (blue) cells.

different bacterial strains, regardless of the coupled yeast strain or the refreshment number considered (Fig. 1, panel A). In particular, the microbial associations with *F. sanfranciscensis* UMB9 and UMB42 strains exhibited, on average, the highest growth rates ($2.94 \pm 0.26 \times 10^{-1}$/h and $2.92 \pm 0.25 \times 10^{-1}$/h, respectively), whereas those with UMB21 strain displayed the lowest one ($1.64 \pm 0.62 \times 10^{-1}$/h). Conversely, the single yeast strain did not affect the μ_m of mixed populations since no differences ($p = 0.755$) were observed. The effect of the refreshment, simulating the back-slopping practice, was significant ($p = 0.031$) whatever the examined co-cultures were, as the overall average of growth rates increased from $2.21 \pm 0.59 \times 10^{-1}$/h (first refreshment) to $2.48 \pm 0.57 \times 10^{-1}$/h (third refreshment) (Fig. 1, panel B).

When the interaction between the "bacterial strain" and the "refreshment" effects has been considered, the ANOVA returned significant differences ($p < 0.001$) between mean values (Table 2); this indicates that some *F. sanfranciscensis* singularly causes a change in growth rate of the respective microbial association during the consecutive re-inoculations. This is the case of UMB21 strain, that increases the μ_m of its mixed populations, on average, from $1.41 \pm 0.12 \times 10^{-1}$/h (first refreshment) to $2.12 \pm 0.12 \times 10^{-1}$/h (third refreshment), as well as UMB49 strain, which pushes the growth rate of its co-cultures from $1.76 \pm 0.12 \times 10^{-1}$/h (first refreshment) to $2.78 \pm 0.12 \times 10^{-1}$/h (third refreshment), both regardless of the associated yeast strain.

The elaboration of estimated λ values revealed that the bacterial strain is a key factor in determining the lag times for microbial consortia. In fact, the mean latency phase of those associations with *F. sanfranciscensis* UMB21 strain (4 h 32 min) was significantly longer ($p < 0.001$) than those with all the other bacterial strains, which ranged from 2 h 19 min for UMB9 to 3 h 4 min for UMB37, irrespectively of yeast strain co-inoculated (Fig. 2, panel A). On the other hand, the role of yeast strain was confirmed not to be significant for the duration of lag times exhibited by the mixed populations ($p = 0.829$), although the λ

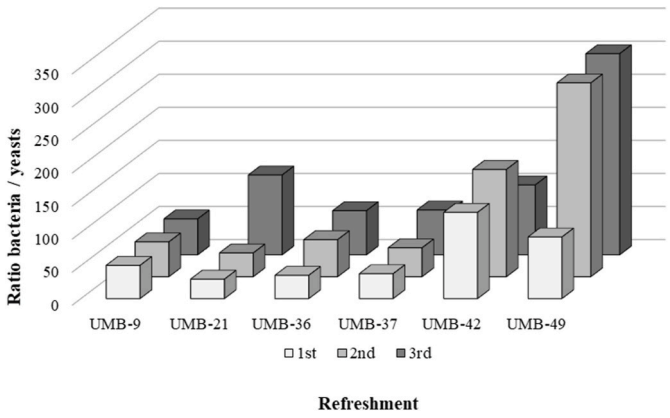

Fig. 4. Trends of ratio bacterial/yeast cells in co-cultivation experiments revealed in consecutive refreshments depending on the *F. sanfranciscensis* strain.

Table 4
Mean values ± standard deviation of the ratio bacterial/yeast cells observed in co-cultivation experiments with different strains of *F. sanfranciscensis* and *K. humilis*.

	Co-cultivated yeast strain					
Bacterial strain	UMY1	UMY9	UMY10	UMY12	UMY98	average
UMB9	53 ± 35	65 ± 28	51 ± 24	59 ± 25	35 ± 16	52^{a} ± 26
UMB21	33 ± 40	81 ± 100	118 ± 190	38 ± 47	40 ± 39	62^{a} ± 99
UMB36	45 ± 38	73 ± 63	32 ± 12	62 ± 26	52 ± 16	53^{a} ± 35
UMB37	40 ± 29	32 ± 15	67 ± 46	64 ± 25	46 ± 22	50^{a} ± 30
UMB42	190 ± 117	155 ± 141	70 ± 33	128 ± 89	121 ± 112	133^{b} ± 105
UMB49	187 ± 131	179 ± 172	174 ± 88	321 ± 222	289 ± 155	230^{c} ± 162
Average	91 ± 100	98 ± 110	85 ± 96	112 ± 136	97 ± 119	

Values with different superscripts letters in the same column are significantly different ($p < 0.001$). Tukey's HSD test was performed with interval confidence at 95%.

values in yeast-free controls lasted more (3 h 24 min), on average, than those with yeasts (2 h 58 min). Finally, a significant impact on latency phase of microbial associations was demonstrated by the operation of sub-culturing ($p < 0.001$) since the mean λ value of first refreshment was shorter (1 h 32 min) than those of the second and third refreshments (4 h 39 min and 3 h 39 min, respectively), regardless of the co-cultivated strains (Fig. 2, panel B). This trend could be explained as the cells of the first inoculum were harvested in the exponential phase, whereas the cells of the second and third refreshments were sub-cultured in the early stationary phase, by mimicking the back-slopping operation.

The ANOVA of λ data has pointed out that the interaction between the "bacterial strain" and the "refreshment" effects can determine significant differences ($p < 0.001$) on the duration of lag times (Table 3). In particular, UMB21 strain elongates the latency phase of its mixed populations, on average, from 2 h 14 min (first refreshment) to 5 h 46 min (second refreshment) and 3 h 44 min (third refreshment), irrespectively to the associated yeast strain. For all the other cases, bacterial strains stabilize the latency times of their corresponding microbial associations, no longer showing significant differences between the second and third refreshments.

Microscopic images (RGB images, 1944 × 2592 × 3) were captured to sort and calculate the numerical proportion between bacterial and yeasts cells at the end of each refreshment. The zoom of the image acquired for UMY1-UMB42 co-cultivation experiments is reported in Fig. 3 (panel A). Firstly, the red channel has been extracted from the RGB image (1944 × 2592 × 3) (Fig. 3B), then it has been converted into binary image by thresholding so that zero values corresponded to background while one values corresponded to pixels attributed to microorganisms (Fig. 3C). To do so the red-channel-image has been smoothed by a convolution filter (5-by-5 pixel based), then morphological operators, i.e. opening and closing algorithms, have been used for object erosion and dilatation (Fig. 3D). To highlight the cells boundaries a minimum filter was applied so that the intensity of each pixel was corrected against the surrounding pixels in a 5-by-5 pixel based running window. Lastly, the image was sharpened by delineation to improve the cell edges definition (Fig. 3E). After the cleaning procedure of the image, the differentiation between LAB and yeast was performed based on the object area in the obtained binary image. The ratio bacterial/yeast cells was expressed as the proportion between small (area <300 pixels) and big (area >300 pixels) objects (Fig. 3F).

The numerical ratios between bacterial and yeast cells at the end of each refreshment for each co-culture was calculated (Fig. 4). The obtained results support the hypothesis that *F. sanfranciscensis* regulates the proportion among the cells within the couple, since significant differences among mean values of numerical ratios were found with different bacterial strains ($p < 0.001$), whereas *K. humilis* by getting no difference ($p = 0.892$) (Table 4). The effect of sub-culturing played a pivotal role for the stabilization of the numerical ratios in some mixed populations, such as those with strains UMB9, UMB36 and UMB49 (Fig. 4). In general, significant differences ($p = 0.012$) were found, on average, between the first refreshment and the last two ones with averaged ratios bacteria/yeast cells of 62, 107 and 120, respectively.

3.2. Comparison of models to predict growth kinetics of the microbial consortia

Experimental growth data obtained by 210 co-cultivation tests and relevant controls have been fitted using Gompertz's modified by Zwietering et al. (1990), Baranyi and Roberts' (1994), and Schiraldi's (2017) functions. All the obtained curves have been deposited and available in UNIMI Dataverse (Foschino et al., 2020). Fig. 5 shows, as exemplification, the curves with the best fitting for each of considered growth models (Panels A, C and E) and those with the worst ones (B, D, F). Surprisingly, all the functions have soundly predicted the growth kinetics for microbial associations, which though do not respect the cell duplication paradigm. Nevertheless, differences were observed in the outcome of fitting operation: when there were many experimental points in the stationary phase, the Schiraldi's function worked better than the other ones, while it behaved worst during the transition period between the lag and exponential growth phases. In order to evaluate the goodness of the fit, the Root Mean Square value was calculated for each growth test by using the three different models, and the relative data are reported in Table 5. The comparison of the results indicates that Schiraldi's function has performed more effectively revealing the smallest RMS values with an overall average of 0.0219, whereas Baranyi and Roberts' model has disclosed the largest ones with an overall average of 0.0382. The Gompertz's function modified by Zwietering behaved in an intermediate way showing an overall average of 0.0282. Schiraldi's model has provided the best solution of fitting for over 70% of co-cultivation experiments, making it consistent for this application.

3.3. Productivity of the strains pairs

Knowing which bacterium and yeast pair has the highest productivity in terms of biomass *per* time is fundamental for starter cultures manufacturers. In this study, the cell biomass production, that has been evaluated as optical density of the sample after 24h, was significantly affected ($p < 0.001$) by the *F. sanfranciscensis* strain present in co-cultivation experiments, but not by *K. humilis* strain ($p = 0.744$). In

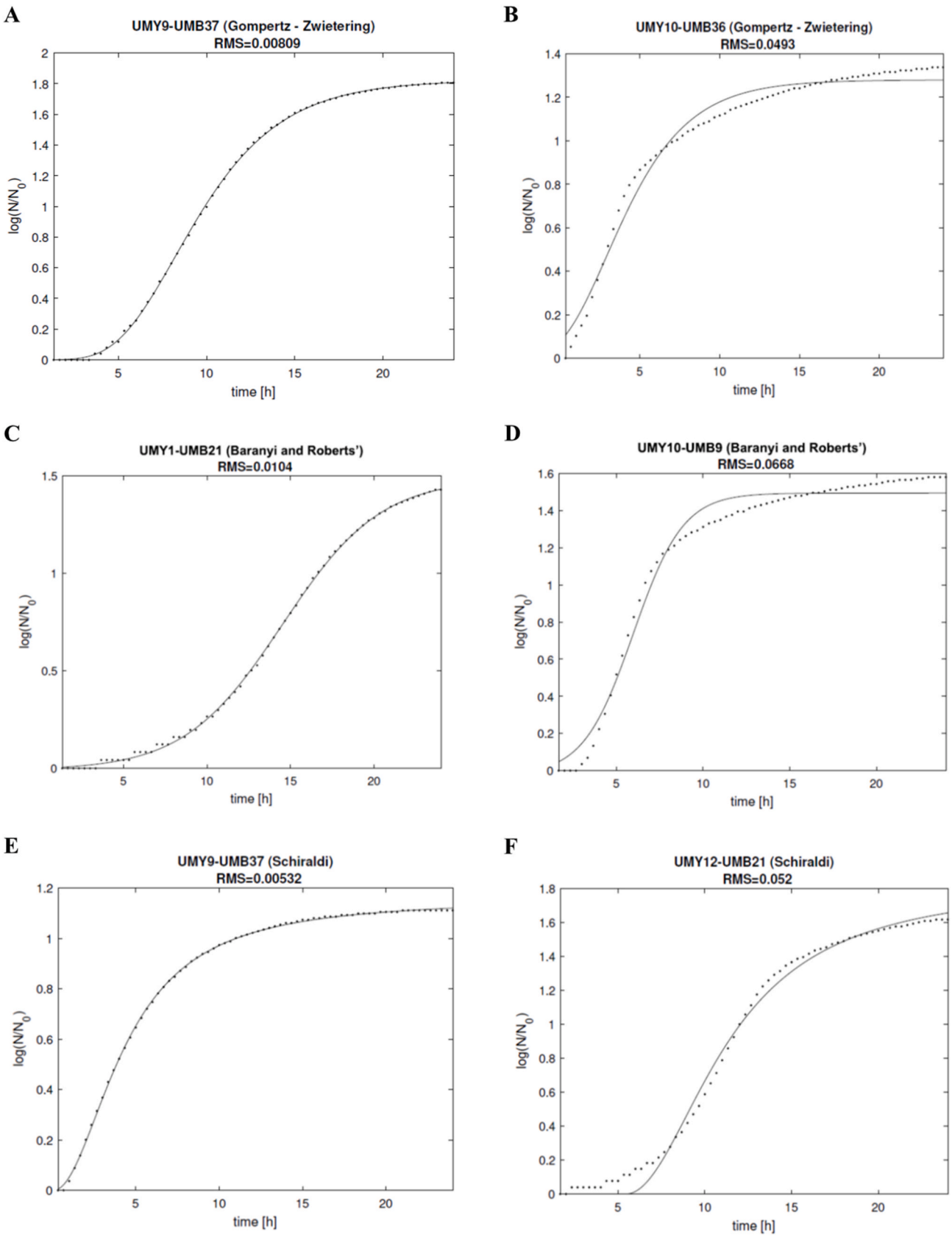

Fig. 5. Comparison of the fitting curves generated by different growth models. The choice of the experimental data related to a specific couple of strains was done on the basis of the lowest values of RMS exhibited with Gompertz's modified Zwietering (A), Baranyi and Roberts' (C) and Schiraldi's (E) functions, and on the basis of the highest values of RMS for Gompertz's modified Zwietering (B), Baranyi and Roberts' (D) and Schiraldi's (F) functions, respectively. Lines represent the model fits, while dots represent the observed data.

Table 5
Average values of Root Mean Square obtained with the three different growth models for each combination of *F. sanfranciscensis* and *K. humilis* strains.

Bacterial strain	Yeast strain	Gompertz mod. Zwietering	Baranyi and Roberts	Schiraldi
UMB9	UMY1	0.0308	0.0416	0.0212
UMB9	UMY9	0.0251	0.0359	0.0227
UMB9	UMY10	0.0389	0.0511	0.0205
UMB9	UMY12	0.0299	0.0416	0.0202
UMB9	UMY98	0.0297	0.0395	0.0216
UMB21	UMY1	0.0239	0.0197	0.0236
UMB21	UMY9	0.0206	0.0285	0.0205
UMB21	UMY10	0.0244	0.0283	0.0224
UMB21	UMY12	0.0285	0.0247	0.0285
UMB21	UMY98	0.0261	0.0280	0.0246
UMB36	UMY1	0.0305	0.0416	0.0215
UMB36	UMY9	0.0231	0.0333	0.0222
UMB36	UMY10	0.0317	0.0447	0.0199
UMB36	UMY12	0.0345	0.0462	0.0159
UMB36	UMY98	0.0330	0.0406	0.0248
UMB37	UMY1	0.0246	0.0393	0.0241
UMB37	UMY9	0.0254	0.0385	0.0218
UMB37	UMY10	0.0240	0.0384	0.0240
UMB37	UMY12	0.0270	0.0415	0.0205
UMB37	UMY98	0.0339	0.0445	0.0257
UMB42	UMY1	0.0314	0.0404	0.0232
UMB42	UMY9	0.0244	0.0331	0.0234
UMB42	UMY10	0.0344	0.0435	0.0250
UMB42	UMY12	0.0318	0.0408	0.0262
UMB42	UMY98	0.0295	0.0371	0.0242
UMB49	UMY1	0.0267	0.0422	0.0176
UMB49	UMY9	0.0186	0.0334	0.0194
UMB49	UMY10	0.0295	0.0440	0.0180
UMB49	UMY12	0.0279	0.0440	0.0169
UMB49	UMY98	0.0274	0.0412	0.0179
Mean		0.0282	0.0382	0.0219

Table 6
Mean values ± standard deviation of final cell biomasses (OD_{600nm}) normalized as log (N/No) observed in co-cultivation experiments with different strains of *F. sanfranciscensis* and *K. humilis*.

	Co-cultivated yeast strain					
Bacterial strain	UMY1	UMY9	UMY10	UMY12	UMY98	average
UMB9	1.22 ± 0.13	1.23 ± 0.20	1.30 ± 0.12	1.20 ± 0.18	1.21 ± 0.11	$1.23^{b,c}$ ± 0.14
UMB21	1.02 ± 0.17	1.19 ± 0.28	1.13 ± 0.15	1.08 ± 0.12	1.18 ± 0.18	1.12^{a} ± 0.18
UMB36	1.41 ± 0.10	1.41 ± 0.17	1.45 ± 0.11	1.47 ± 0.13	1.35 ± 0.19	1.42^{d} ± 0.13
UMB37	1.50 ± 0.06	1.43 ± 0.13	1.49 ± 0.06	1.51 ± 0.08	1.38 ± 0.09	1.46^{d} ± 0.10
UMB42	1.14 ± 0.16	1.11 ± 0.19	1.17 ± 0.17	1.12 ± 0.16	1.14 ± 0.16	$1.14^{a,b}$ ± 0.16
UMB49	1.33 ± 0.06	1.25 ± 0.11	1.37 ± 0.06	1.32 ± 0.09	1.27 ± 0.11	1.31^{c} ± 0.09
average	1.27 ± 0.20	1.27 ± 0.19	1.31 ± 0.15	1.28 ± 0.19	1.25 ± 0.14	

Values with different superscripts letters in the same column are significantly different ($p < 0.001$). Tukey's HSD test was performed with interval confidence at 95%.

details, the highest optical density values were achieved by the microbial association in which UMB36 and UMB37 strains were used (Table 6). Noteworthy, both showed an intermediate behaviour for μ_m and λ in co-cultures, thus demonstrating that values of these parameters have not a proportional causal relationship with the productivity of the couple. In addition, these bacterial strains together with UMB9, definitely stabilized their numerical ratio with yeasts around 50, even if they demonstrate to have dissimilar growth rates. As regards *K. humilis*, microbial associations with UMY10 yeast strain performed better than the others, although mean values were not significantly different. As expected, cell biomass values in control test experiments (single bacterial strains) have always been lower than the respective pairs in co-cultivation (data not shown).

This outcome makes it convincible the choice of producing a starter as a mixed culture starting from a co-inoculum to be fixed on the numerical ratio (bacterial/yeast cells) which stabilizes between the considered strains.

4. Discussion

While co-cultivation is considered the most efficient way for biomass production and stabilization of the balance between microbial species (Bader et al., 2010), the available information on growth kinetics of sourdough microorganisms is very limited (Gianotti et al., 1997; Neysen and De Vuyst, 2005); only one paper described the modelling of growth of *Lactobacillus sanfranciscensis* and *Candida milleri* specifically in response to process parameters of sourdough fermentation (Gänzle et al., 1998).

The first purpose of this work has aimed at filling the information gap about the role of single bacterial and yeast strain in sourdough consortia and at identifying the most productive combination between them within a limited microbial collection for starter cultures production. Indeed, although the microbiological characterization of sourdough communities has been extensively documented in literature by diverse approaches, for different practiced technologies and in ecological studies, the strain-level interactions have been poorly investigated. Recently, the relationship at strain level between *K. humilis* and LAB has been described in sourdough by Carbonetto et al. (2020); their results have demonstrated that in co-cultures LAB are not influenced by the presence of the yeast strain and *K. humilis* does not take advantage in terms of population size from the association. The authors question the most accredited hypothesis based on the positive interactions between the sourdough microbial species, as widely reported in bibliographic references (Introduction paragraph), and they argue that not mutualism, but competition would rule the co-existence of microorganisms in the sourdough environment too. Furthermore, Rogalski et al. (2020) have recently published a very interesting study on intra-species interactions of *F. sanfranciscensis* in rye sourdoughs showing that the competitiveness of strains is a strain-specific trait, and it can depend or not on the presence of the yeast species. Our observations confirm this new supposition, since the single *F. sanfranciscensis* strain was the regulating factor for the growth parameters and the proportion among cells in the sourdough microbial association, regardless of the co-cultivated yeast strain. Moreover, the maximum yield in terms of whole biomass of the couple is not determined by the specific maximum growth rate of the involved bacterial strain, but could be related to the stabilization of the numerical ratio, inside strain specificity. In other words, the fastest strain may be the most performant at achieving dominance, but not the one for which the highest density of bacterial and yeast cells is obtained. This probably implies that the sourdough microorganisms can undergo not only to metabolic interactions for stabilizing the consortium (Ehrmann and Vogel, 1998; Gobbetti, 1998), but might also communicate with each other to control population density with other mechanisms, like those observed in *quorum sensing* (Bader et al., 2010; Smid and Lacroix, 2013). The capacity of *F. sanfranciscensis* to interact with other lactobacilli on the basis of bacterial cell–cell communication by secretion and detection of inducer molecules has been investigated by Calasso et al. (2013), but no work has yet been published on interaction with sourdough yeasts. A deepening of knowledge on this issue should be addressed.

Finally, it should be noted that the choice of using a cultural broth and not a mixture of water and flour has been made to envisage a possible development of growth medium for biomass production and to be able to continuously monitor the cell growth by spectrophotometric readings. As regards the second purpose, we implemented a "learning

machine" approach to achieve the comparison among the results obtained from two most used microbial growth models (Gompertz's modified by Zwietering et al., 1990; Baranyi and Roberts, 1994), and that of Schiraldi (2017): the chosen algorithm, based on an iteration process, makes subsequent predictions to minimize the errors respect to the observed values to determine which model fits best to experimental data. Some mathematical functions are reliable tools currently applied in predictive microbiology for estimating the growth kinetics of single strains that carry out cell duplication. The new model proposed by Schiraldi seems to be able to overcome this paradigm since it takes into account the system convolution due to the asynchronous behaviors of individual cells in the culture. While the contribution of individual cell lines is no longer decipherable, this makes possible to apply the model in mixed populations, as we have shown. Actually, all three tested primary functions fit well showing RMS values lower than those found by Buchanan et al. (1997) for Gompertz's and Baranyi's equations with other set of data. Thus, the presence of two co-cultivated microorganisms and the mode of cell replication (binary fission or budding) do not affect the capacity of the models to consistently predict the growth kinetics of the association. Finally, this can pave the way for better understanding the balance within microbial consortia and for controlling and improving fermentation processes carried out with mixes cultures in industrial applications (Bader et al., 2010; Smid and Lacroix, 2013).

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Declarationof competing interest

All authors of the manuscript entitled "Investigating the growth kinetics in sourdough microbial associations" state that there is no conflict of interests to declare.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fm.2021.103837.

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