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Modelling sequential co-inoculation of a non-conventional biocontrol yeast and *Saccharomyces cerevisiae* in wine

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ABSTRACT

Microbial interactions play a decisive role in fermentation dynamics and final wine quality. The sequential co-inoculation of non-conventional yeasts with *Saccharomyces cerevisiae* has been proposed as a strategy to enhance organoleptic properties, reduce ethanol content and/or exert biocontrol over spoilage yeasts, thereby decreasing the use of sulphur dioxide (SO_2). Among these species, *Wickerhamomyces anomalus* has shown effectiveness; however, its interaction with *S. cerevisiae* under sequential inoculation schemes has not been explored from an ecological modelling perspective. In this study, the population dynamics of both species were analysed using an Ordinary Differential Equation (ODE)-based model, evaluating the influence of co-inoculation timing, temperature, and SO_2 concentration to maximise the viability of the biocontrol yeast without affecting the fermentative kinetics of *S. cerevisiae*. Fifteen fermentations were carried out following a Box–Behnken experimental design, varying temperature (15–20 °C), molecular SO_2 (0–0.2 ppm), and co-inoculation time (0–48 h). A Gilpin–Ayala competition model was applied, incorporating secondary temperature models (Arrhenius and Ratkowsky) and constant parameters for the effects of SO_2 and co-inoculation time. The model was refined through parameter estimation and the Akaike Information Criterion (AIC), and was experimentally validated under different inoculum proportions. The iterative fitting process led to a simplified Lotka–Volterra model modified solely to include the effect of co-inoculation time. Inoculating *S. cerevisiae* 48 h after *W. anomalus* significantly increased the viability of the latter (by 75 % compared to simultaneous inoculation) without affecting fermentative kinetics. Simulations confirmed the final dominance of *S. cerevisiae* and the convergence of both populations toward the same sink point. Delaying *S. cerevisiae* inoculation favoured the early establishment of *W. anomalus*, enhancing biocontrol efficacy without compromising alcoholic fermentation. This simplified ecological model provides a framework to optimise microbial management in winemaking, reduce SO_2 usage, and promote more sustainable fermentation processes.

KEYWORDS: sequential co-inoculation, biocontrol, *Wickerhamomyces anomalus*, ecological modelling

INTRODUCTION

Microbial interactions are highly relevant in oenology, as they shape the biodiversity of the fermentation process and, consequently, the organoleptic characteristics of wine (De Gioia *et al.*, 2022). Within this microbial diversity, certain spoilage yeasts can cause sensory deviations and negatively impact wine quality (Mehlomakulu *et al.*, 2014; Rojo *et al.*, 2015; Kuchen *et al.*, 2019). Traditionally, sulphur dioxide (SO_2) has been used in winemaking to control these undesirable populations; however, its use is increasingly discouraged due to its adverse effects on human health (Vally *et al.*, 2009; Relaño de la Guía *et al.*, 2025).

Biocontrol, understood as the ability of one organism to prevail, dominate, or biosuppress another, has long been proposed as an alternative strategy (Baker & Cook, 1974) and remains highly relevant today, particularly as a means of reducing the use of SO_2 in winemaking (Berbegal *et al.*, 2017; Canonico *et al.*, 2023a; Canonico *et al.*, 2023b). It is an anthropic approach based on natural microbial interaction mechanisms (Boynton, 2019) and is designed according to the technological objectives of the process. For example, in the context of wine fermentation, *Wickerhamomyces anomalus* has been successfully applied against *Zygosaccharomyces rouxii* during the pre-fermentative stage (Kuchen *et al.*, 2023a; Kuchen *et al.*, 2024). Other species, such as *Metschnikowia pulcherrima* and *Torulaspora delbrueckii*, have also been proposed for use in the same phase (Canonico *et al.*, 2023a; Canonico *et al.*, 2023b). Nevertheless, biocontrol yeasts should not alter the conditions required for the establishment and fermentation kinetics of the fermentative yeast (Albertin *et al.*, 2017). Achieving this dynamic balance between both species is crucial to combine biocontrol efficacy with the technological stability of the wine.

Among the factors that promote a favourable environment for biocontrol, the priority effect stands out, because it describes how the order and timing of species establishment shape subsequent interactions (Stroud *et al.*, 2024). In this sense, the sequential co-inoculation of yeasts, such as *Wickerhamomyces anomalus* and *Saccharomyces cerevisiae*, represents a controlled application of the priority effect. By adjusting the inoculation timing and population ratios, it is possible to guide competitive interactions and define the outcome of the process, promoting biocontrol, fermentation stability, or the formation of desirable metabolites (Gallo *et al.*, 2024). This strategy has been extensively studied in oenology and remains an active field of research (Toro & Vázquez, 2002; Su *et al.*, 2024). However, sequential co-inoculation may present challenges related to interspecies competition, which could slow down fermentation, if not properly managed (Albertin *et al.*, 2017). These interactions can also be modulated by physicochemical factors and interventions in the fermentation process, such as temperature and the presence of SO_2, which directly influence growth rate and the maximum population density achieved (Savadó *et al.*, 2011; Baiano *et al.*, 2012; Balsa-Canto *et al.*, 2020). Such factors affect resource competition and the production of primary and secondary metabolites with inhibitory potential (Pommier *et al.*, 2005; Mazzuco *et al.*, 2019; Kuchen *et al.*, 2023b; Georgescu *et al.*, 2024), as well as the stability of these compounds in the medium and the sensitivity of the sensitive yeast (Kuchen *et al.*, 2021).

The modelling of microbial interactions represents the first step in applying process engineering to biotechnology and the wine industry. Although grape must constitutes an ecologically complex system (Lax & Gore, 2023), and more general frameworks are sometimes required to describe microbial communities (Ruiz *et al.*, 2023), this study aims to quantitatively elucidate the interaction mechanisms that emerge during sequential co-inoculation within the interaction dynamics. Mechanistic primary models can be used to determine the magnitude of interesting response variables, such as the maximum specific growth rate or the time required to reach a given level – in this case, one or more interacting populations (Schoener, 1973; Gilpin & Ayala, 1973). However, the parameters growth rate, carrying capacity and interspecific competition can depend on environmental conditions, and secondary models describe this dependence (McMeekin *et al.*, 2002), thereby enabling predictions under untested conditions (Hellweger *et al.*, 2016; Balsa-Canto *et al.*, 2020). Evaluating yeast growth kinetics while simultaneously considering multiple physicochemical factors can be both laborious and costly. Therefore, the use of experimental designs that minimise the experimental workload while maximising the information yield is particularly relevant (Pedrozo *et al.*, 2024).

Few studies have explored sequential co-inoculation in winemaking through ecological interaction models, and those available focuses exclusively on *Saccharomyces* species (Balsa-Canto *et al.*, 2020). To our knowledge, mathematical modelling approaches have not yet been applied to investigate the timing of co-inoculation and other physicochemical factors between non-conventional yeasts and *S. cerevisiae* with the aim of extending biocontrol effects during the early stages of winemaking without affecting the fermentation process itself.

The objective of this study was to evaluate the relevant physicochemical factors and the interaction kinetics of sequential mixed co-inoculation between the biocontrol yeast *W. anomalus* and *S. cerevisiae* from an ecological modelling perspective. Specifically, two main goals were pursued: to maximise the presence of the biocontrol yeast in the medium and to ensure that the fermentative kinetics of *S. cerevisiae* would remain unaffected.

MATERIALS AND METHODS

1. Experimental design

A Box–Behnken design (Fisher, 1935) was employed with three replicates at the central point, totalling 15 experiments. This design allowed the simultaneous evaluation of three factors: temperature (15, 17.5, and 20 °C), SO_2 concentration (0, 0.1, and 0.2 ppm of molecular SO_2),

and sequential co-inoculation time (0, 24, and 48 h). The response variables analysed were the growth kinetics (for 120 h) of the two yeast populations involved: the biocontrol strain *Wickerhamomyces anomalus* BWa156 and the fermentative strain *Saccharomyces cerevisiae* V22 previously identified at the molecular level (Kuchen *et al.*, 2019; Petrignani *et al.*, 2024).

2. Experimental procedure

Pre-inoculum: the yeast strains were activated on YPD-agar. Pre-inoculum of both strains were prepared in 250 mL Erlenmeyer flasks containing 200 mL of concentrated/diluted must adjusted to 21 °Brix and supplemented with 1 % yeast extract. Cultures were incubated for 24 h at 110 rpm and 25 °C.

Fermentation trials: the 15 fermentations were conducted in 250 mL Erlenmeyer flasks containing 200 mL of concentrated/diluted must adjusted to 21 °Brix and supplemented with 0.1 % yeast extract. The initial inoculum for both strains was set at 1.5×10^6 cells/mL. Sampling was performed after 24, 48, 72, 96 and 120 h respectively, in addition to the initial inoculation of both yeast strains; *i.e.*, there was a total of six sampling time points per experiment. Given there were 15 experiments and two yeast species, a total of 180 data points were collected. Yeast counts were conducted on WLN agar plates using appropriate dilutions to ensure colony counts of between 50 and 250 CFU per plate.

3. Mathematical modelling

Two models based on the Lotka–Volterra conceptual framework were proposed to predict the population density of both species in mixed cultures. This model describes intra-and inter-specific competition (Murray, 2001). Intraspecific competition is defined as competition among individuals of the same population for the exploitation of resources or space (Schoener, 1973), while interspecific competition refers to facilitative or inhibitory interactions between populations (Toju *et al.*, 2018). However, Gilpin and Ayala (1973) highlighted the limitations of the Lotka–Volterra framework when dealing with nonlinear patterns of intraspecific regulation.

All these models can be embedded in the following mathematical formulation:

$$\frac{dxi}{dt} = G(xi, xj) = x_i\mu_i * (1 - (\frac{x_i}{K_i})^{\theta_i} - \alpha_{i,j}(\frac{x_j}{K_i}))i, j = Wa, Sc \; i \neq j \quad (1)$$

where i is either *S. cerevisiae (Sc)* or *W. anomalus (Wa)* and j either *Wa* or *Sc* with $i \neq j$; x_i corresponds to the cell density for both species; μ_i corresponds to the specific growth rate; K_i is the carrying capacity for species i; and $\theta_i \geq 1$ controls the degree of non-linearity in intraspecific growth regulation. Coefficients α_{ij} measure the competitive strength of species j on i. If $\alpha_{i,j} > 0$ species or strains are said to be competing.

4. Secondary models

Based on previous data on the dependence of μ on temperature (Salvadó *et al.*, 2011), two secondary models with a maximum of two parameters each were proposed: the Ratkowsky or square root model (SQRT) (Ratkowsky *et al.*, 1983) (Equation 3) and the Arrhenius equation (Equation 4):

$$\mu_i(T) = \mu_{i,0}^2(T - \mu_{i,1})^2 \quad (2)$$

$$\mu_i(T) = \mu_{i,0} * e^{\left(-\frac{\mu_{i,1}}{T}\right)} \quad (3)$$

where, in Ratkowsky, $\mu_{i,j,0}$ is the sensitivity of the response to temperature, and $\mu_{i,j,1}$ is the minimum growing temperature. In Arrhenius, $\mu_{i,j,0}$ is the maximum growing temperature while, $\mu_{i,j,1}$ is the sensitivity. Note that the Arrhenius equations can also explain the case in which the biological parameter is constant with the temperature (with $\mu_{i,j,1} = 0$).

The other two factors, sequential co-inoculation time and SO_2 concentration, were included as constants. The first, *timeCo*, defines the presence or absence of the yeast *S. cerevisiae*. The second, β_1 and β_2, quantifies the sensitivity of each strain to molecular SO_2, acting as a secondary mortality rate.

The resulting system of equations is:

$$\frac{dx_{sc}}{dt} = timeCo * x_{sc} * \mu_{sc}(T) * \left(1 - \left(\frac{x_{sc}}{K_{sc}}\right)^{\theta sc} - \alpha_2 * \frac{x_{wa}}{K_{sc}}\right) - \beta_1 * SO_2 \quad (4)$$

$$\frac{dx_{wa}}{dt} = x_{wa} * \mu_{wa}(T) * \left(1 - \left(\frac{x_{wa}}{K_{wa}}\right)^{\theta wa} - \alpha_1 * \frac{x_{sc}}{K_{wa}}\right) - \beta_2 * SO_2 \quad (5)$$

5. Theoretical methods: the modelling process

Modelling was approached from a system identification perspective, which included the following steps: formulation of candidate models, parameter estimation from multiple experiments, and model reduction and selection.

6. Formulation of candidate models

The Gilpin–Ayala model was employed as the initial candidate owing to its higher level of complexity and considering the biology of the competition system (primary model) and different phenomenological submodels to account for temperature. The model solution depends on the given initial conditions, and the values of a series of unknown parameters.

7. Parameter estimation

The aim was to calculate the unknown parameters, constants related to growth, and kinetic parameters that minimise the distance between the data and the model predictions, in other words, the error.

One way to minimise the distance is through an objective function that includes the measure of distance using least squares:

$$J(\theta) = \frac{1}{2}\sum_{i=1}^{n_{exp}}\sum_{j=1}^{n_{obs(i)}} r_{ij}(\theta)^T Q_{expmax,ij} r_{ij}(\theta) \quad (6)$$

where n_{exp} is the number of experiments and n_{obs} is the number of observables. For each experiment and residuals are given by:

$$r_{ij}(\theta) = y_{ij} - \tilde{y}_{ij}(\theta) \qquad (7)$$

here, $y_{ij} \in \mathbb{R}^{n_{t,ij}}$ are the experimental data samples for observable j in experiment i, and $\tilde{y}_{ij}(\theta) \in \mathbb{R}^{n_{t,ij}}$ are the corresponding model predictions. The parameter vector is denoted by $\theta \in \mathbb{R}^{n_\theta}$.

Instead of using the identity matrix as weight, we adopted a normalisation strategy based on the maximum experimental value per observable and experiment:

$$Q_{expmax,ij} = \frac{1}{(y_{ij}^{max})^2} I, \quad y_{ij}^{max} = max_k y_{ij}(t_k) \qquad (8)$$

This choice scales the residuals by the maximum amplitude observed y_{ij}^{max} in each time series t_k, ensuring a comparable contribution across observables with different units or magnitudes.

8. Model selection and reduction

The models were iteratively compared based on their ability to fit the experimental data. Since models with more parameters often have better fits but tend to overfit, the number of parameters was also considered. The Akaike Information Criterion (AIC) was used for this purpose, aiming to balance parsimony and the relative loss of information among candidate models by penalising the number of parameters (Burnham & Anderson, 2004). For least squares is defined as follows:

$$AIC_M = nd * \log\left(\frac{RSS}{nd}\right) + 2np + 1 \qquad (9)$$

where, *np* is the number of parameters, *nd* is the number of data points, and *RSS* is the sum of squared residuals. The process started with the most complex candidate models, and after fitting the data, less influential parameters were iteratively eliminated and evaluated through the AIC strategy.

The minimum AIC_M value AIC_{min} was used to rescale *AIC*. The rescaled value $\Delta M = AIC_M - AIC_{min}$ was used to measure the model's merit: models with $\Delta M \leq 2$ provide substantial support, models with $4 \leq \Delta M \leq 7$ provide considerably less support, and models with $\Delta M > 10$ have no support (Burnham & Anderson, 2004).

9. Numeric tools

To automate the modelling process, we used the AMIGO2 toolbox (Balsa-Canto *et al.*, 2020). AMIGO2 is a MATLAB-based tool focused on the identification and optimisation of parametric models, including sensitivity and identifiability analysis. It provides a set of numerical methods for both simulation and optimisation. From the available options, we selected CVODES (Hindmarsh *et al.*, 2005) to solve the model equations and enhanced Scatter Search (eSS) (Egea *et al.*, 2009) to find optimal parameter values within a reasonable time frame.

10. Initial inoculum simulations

The final model was used to develop simulations for the different co-inoculation times: 0, 24, and 48 h. The simulations (400) were performed using the Runge–Kutta fourth-order method, based on varying initial inoculums from 5×10^6 to 1×10^8 cells/mL of each yeast isolate.

11. Simulation validation

Model predictions were validated under experimental midpoint conditions (0.1 ppm molecular SO_2 and 17.5 °C). Validation was performed using three assays in which the initial inoculum concentrations were set to 5×10^6 cells/mL of *W. anomalus* and 5×10^5 cells/mL of *S. cerevisiae*, applied at sequential co-inoculation times of 0, 24, and 48 h.

RESULTS AND DISCUSSION

Mixed sequential inoculation is sought for various purposes: to enhance the organoleptic profile (Albertin *et al.*, 2017), reduce ethanol concentration (Maturano *et al.*, 2019), or biocontrol spoilage microorganisms (Canonico *et al.*, 2023a; Canonico *et al.*, 2023b). According to our literature review, this work represents an application of classical population dynamics modelling to the oenological context, specifically addressing co-inoculation processes for spoilage yeast biocontrol and their possibly influencing factors.

1. Obtained kinetics

Figure 1 presents the 15 kinetics of mixed sequential co-inoculation of *Wickerhamomyces anomalus* and *Saccharomyces cerevisiae*. These kinetics were conducted based on a Box–Behnken design, varying the initial conditions of co-inoculation times (0, 24, and 48 h), molecular SO_2 concentrations (0, 0.1, and 0.2 ppm), and temperatures (15, 17.5, and 20 °C).

The kinetics were developed up to day 6, as *W. anomalus* is expected to be present during this pre-fermentation period, contributing to biocontrol and even to the organoleptic profile, before significantly decreasing in population (Kuchen *et al.*, 2024). Moreover, as a facultative aerobic yeast (Mehlomakulu *et al.*, 2021), its persistence is strongly influenced by oxygen concentrations in the medium, which, under conditions of strong and predominant kinetics of *S. cerevisiae*, would force it to shift its metabolism to a less efficient state for this microorganism (Qin *et al.*, 2024).

2. Model fitting

A total of n = 180 data points were used to fit the models. The Gilpin–Ayala model with submodels, described in the system of equations (3–6), was evaluated.

2.1. Parameter screening

The influence of temperature on the specific growth rate parameter was evaluated using submodels applied in previous studies (Salvadó *et al.*, 2011; Balsa-Canto *et al.*, 2020). Each best fit was obtained using a combination of Arrhenius or Ratkowsky submodels (Equations 3 and 4) (Ratkowsky *et al.*, 1983), applied to describe the maximum specific growth rate of each species (Table 1). This evaluation was performed based on the goodness of fit for each combination of submodels with the Gilpin–Ayala model modified by us (Equations 5 and 6).

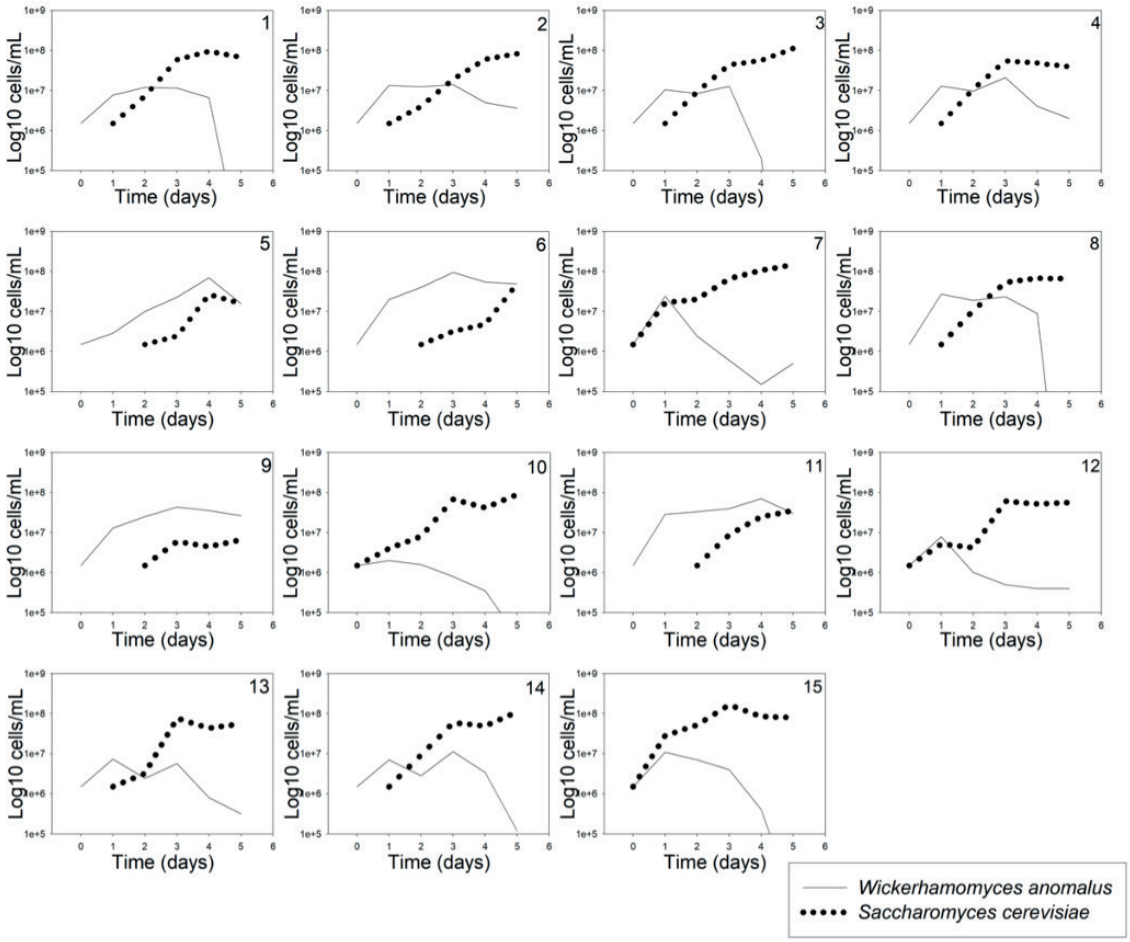


FIGURE 1. Fifteen kinetics of mixed sequential co-inoculation of *Wickerhamomyces anomalus* and *Saccharomyces cerevisiae*. Treatments with variable initial conditions (temperature, SO_2, and co-inoculation time), following Box–Behnken design: treatment 1: 17.5 °C, 0.1 ppm, 24 h. 2: 15 °C, 0 ppm, 24 h. 3: 15 °C, 0.2 ppm, 24 h. 4: 17.5 °C, 0.1 ppm, 24 h. 5: 15 °C, 0.1 ppm, 48 h. 6: 17.5 °C, 0 ppm, 48 h. 7: 17.5 °C, 0 ppm, 0 h. 8: 20 °C, 0 ppm, 24 h. 9: 17.5 °C, 0.2 ppm, 48 h. 10: 15 °C, 0.1 ppm, 0 h. 11: 20 °C, 0.1 ppm, 48 h. 12: 20 °C, 0.1 ppm, 0 h. 13: 17.5 °C, 0.1 ppm, 24 h. 14: 15 °C, 0.2 ppm, 24 h. 15: 17.5 °C, 0.2 ppm, 0 h.

The model incorporating both Arrhenius-type submodels showed a better fit than the alternatives, consistent with its widespread use to describe the effect of temperature on biological parameters (Balsa-Canto *et al.*, 2020). However, as $\mu_{i,l} = 0$ the resulting exponential terms were equal to one, reducing the response "temperature" to a constant value and leading to the submodel removal. Biologically, this outcome is attributed to the narrow experimental temperature range (15–20 °C), initially considered representative of white wine fermentation (Petrignani *et al.*, 2024). However, this choice limited the interpretation of the thermal effect on yeast interactions, constituting a relevant constraint of the study, especially considering red wine fermentation (Kuchen *et al.*, 2021). From a mathematical standpoint, the Ratkowsky model describes the basal response of an organism during the phase preceding the increase in its growth rate, whereas the Arrhenius equation reflects its behaviour as it approaches the maximum growth rate. This suggests that the evaluated conditions are close to the optimal range for both yeasts, which can be considered low-temperature mesophiles, consistent with the findings of Petrignani *et al.* (2024) for the V22 strain.

The reduced model, without temperature influence (submodels), was fitted using 10 parameters (Table 2). Intraspecific competition θ, occurs within the same population, and can exhibit nonlinearity due to population density and resource consumption effects (Schoener, 1973). However, the estimated parameters converged to 1, indicating linear inhibition consistent with the original Lotka–Volterra formulation; therefore, the θ terms were removed. It may be possible to explain this outcome by the nutrient abundance in the must during the six-day fermentation period of this study, which likely prevented the occurrence of resource competition and, consequently, the detection of intraspecific inhibition effects (Fronhofer *et al.*, 2024).

On the other hand, the parameters associated with SO_2, β_1, and β_2 converged to zero, indicating that it had no significant effect on the growth of either yeast. This could be attributed to the low concentration tested (0.2 ppm) and the known tolerance of *W. anomalus* (up to 0.4 ppm; Kuchen *et al.*, 2019) and *S. cerevisiae* V22 to this compound (0.51 ppm; Petrignani *et al.*, 2024). Although these limits were qualitatively known, it was assumed that a lower

concentration would reveal changes in the kinetics, which was not observed. Therefore, this experimental choice represents another relevant limitation of the study.

Then, the Lotka–Volterra model was fitted considering only the co-inoculation time as a factor ($R^2 = 0.774$). Both interaction parameters α were positive, indicating competing organisms (Table 2). The value of α_2, representing the effect of *W. anomalus* on *S. cerevisiae*, was close to zero, indicating a minimal influence of the biocontrol yeast on *S. cerevisiae*. By contrast, α_1, on the order of unity, revealed that *S. cerevisiae* exerted strong competition over *W. anomalus*, indicating a predominantly unidirectional interaction.

These results show that the temporal sequence of inoculation significantly influenced the overall dynamics of *W. anomalus*. Such behaviour could be interpreted within the framework of the priority effect, defined as the phenomenon by which colonisation success and population dynamics, in this case *W. anomalus,* depend on the order of arrival to a local site or niche (Stroud *et al.*, 2024). The inhibition of *W. anomalus* by *S. cerevisiae* could be attributed, at least in part, to competition for oxygen as a limiting resource. Since *W. anomalus* is a facultative aerobic yeast (Mehlomakulu *et al.*, 2021), reduced oxygen availability would affect its metabolism and decrease its population. This effect could be further intensified by oxygen consumption by *S. cerevisiae*, limiting its availability to *W. anomalus* and reflecting potential competition for this resource (Hagman & Piškur, 2015).

For simplicity, robustness, and applicability, the interaction parameter of *W. anomalus* on *S. cerevisiae* α_2 was removed, yielding a fit of 0.781, which is slightly lower than the classical Lotka–Volterra model. The fitted parameters are shown in Table 2, and the model provides a good description of the data. Nevertheless, the comparison using the Akaike Information Criterion (AIC) (Table 3), performed to avoid overparameterisation, indicated low relevance of the physicochemical factors, except for co-inoculation time, and no improvement after removing α_2; therefore, it was retained in the model.

2.2. Debugged model

$$\frac{dx_{sc}}{dt} = timeCo * x_{sc} * \mu_{sc} * \left(1 - \left(\frac{x_{sc}}{K_{sc}}\right) - \alpha_1 * \frac{x_{wa}}{K_{sc}}\right) \quad (10)$$

$$\frac{dx_{wa}}{dt} = x_{wa} * \mu_{wa} * \left(1 - \left(\frac{x_{wa}}{K_{wa}}\right) - \alpha_2 * \frac{x_{sc}}{K_{wa}}\right) \quad (11)$$

TABLE 1. Submodels used and goodness of fit.

Parameter	Submodels combination			
μ_{sc}	Arrhenius	Ratkowsky	Arrhenius	Ratkowsky
μ_{wa}	Arrhenius	Arrhenius	Ratkowsky	Ratkowsky
Best fit	0.77	1.11	1.48	1.91

TABLE 2. Parameter estimation, uncertainty, and screening summary.

Gilpin–Ayala models						Lotka–Volterra models					
Parameter	Optimal value	Uncertainty	Parameter	Optimal value	Uncertainty	Parameter	Optimal value	Uncertainty	Parameter	Optimal value	Uncertainty
$\mu_{sc,0}$	0.049	129 %	μ_{sc}	0.051	129 %	μ_{sc}	0.054	35.10 %	μ_{sc}	0.0453	21.40 %
$\mu_{sc,1}$	0	Inf %	μ_{wa}	0.033	178 %	μ_{wa}	0.034	37.70 %	μ_{wa}	0.0302	26.40 %
$\mu_{wa,0}$	0.028	111 %	α_1	1.92	160 %	α_1	1.55	125 %	α_1	2.78	101 %
$\mu_{wa,1}$	0	Inf %	α_2	0.012	359 %	α_2	0.0019	215 %	K_{sc}	7.64	2.01 %
α_1	1.92	160 %	K_{sc}	7.66	6.69 %	K_{sc}	7.66	2.12 %	K_{wa}	7.81	4.77 %
α_2	0.011	359 %	K_{wa}	7.68	13.10 %	K_{wa}	7.61	5.50 %			
K_{sc}	7.66	6.69 %	θ_{sc}	1	176 %						
K_{wa}	7.68	13.10 %	θ_{wa}	1	219 %						
θ_{sc}	1.12	176 %	β_1	0	Inf %						
θ_{wa}	1.25	219 %	β_2	0	Inf %						
β_1	1.03	120 %									
β_2	2.01	211 %									

TABLE 3. Akaike information criterion for model evaluation.

Model	Gilpin–Ayala (*timeCo*, temperature, SO_2)	Gilpin–Ayala (*timeCo*, SO_2)	Lotka–Volterra	Lotka–Volterra unidirectional
	12	10	6	5
AICc	–230.3678	–242.4008	–251.9148	–252.4124
ΔAICc	12.033	10.0116	0.4976	0
	No support	No support	No considerably less support	Reduced model

2.3. Simulated kinetics

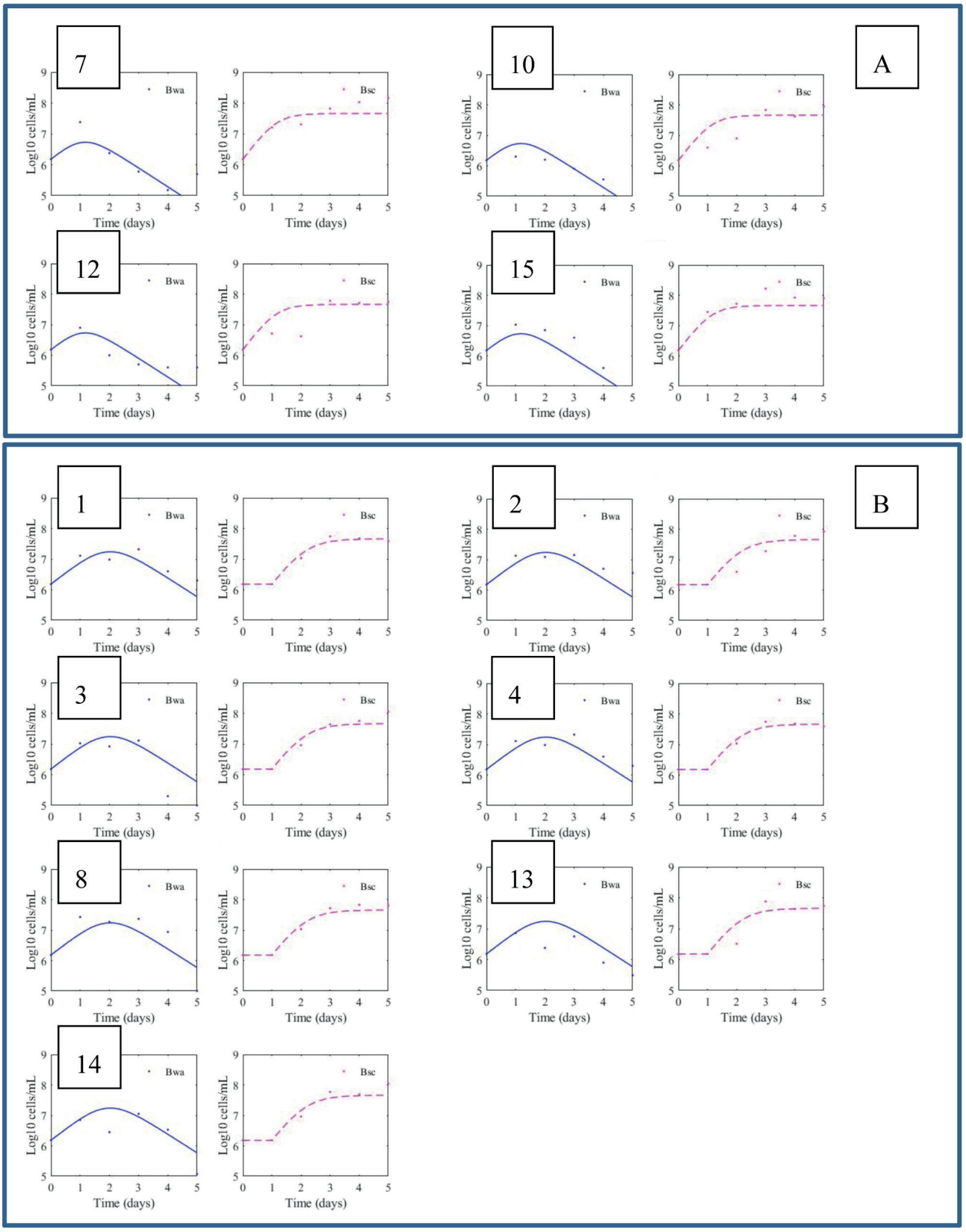


FIGURE 2. Data and model simulation curves: treatments with variable temperature and SO_2: A) Co-inoculation time: 0 h. Treatments: 7: 17.5 °C – 0 ppm. 10: 15 °C – 0.1 ppm. 12: 20 °C – 0.1 ppm. 15: 17.5 °C – 0.2 ppm. B) Co-inoculation time: 24 h. Treatments: 1: 17.5 °C – 0.1 ppm. 2: 15 °C – 0 ppm. 3: 15 °C – 0.2 ppm. 4: 17.5 °C – 0.1 ppm. 8: 20 °C – 0 ppm. 13: 17.5 °C – 0.1 ppm. 14: 15 °C – 0.2 ppm. C) Co-inoculation time: 48 h. Treatments: 5: 15 °C – 0.1 ppm. 6: 17.5 °C – 0 ppm. 9: 17.5 °C – 0.2 ppm. 11: 20 °C – 0.1 ppm. (part 1/2)

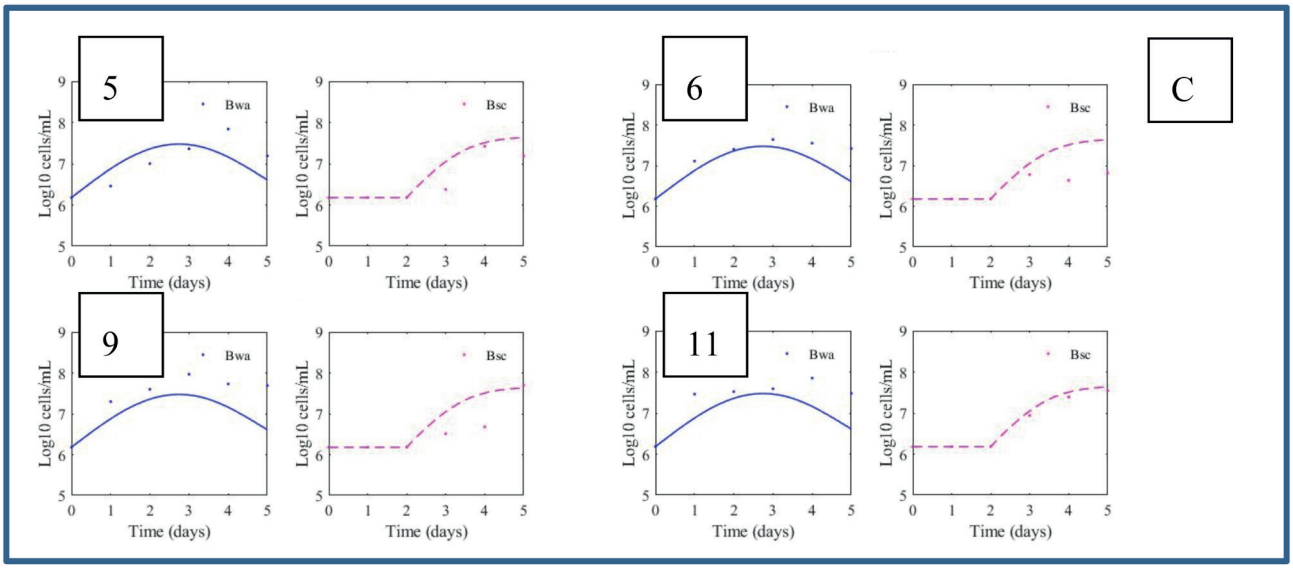


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2.4. Correlation analysis

There is a correlation between the interaction parameter of *Saccharomyces cerevisiae* on *W. anomalus* α_1 and the carrying capacity of the biocontroller yeast *Kwa* (Figure 3). This fact, could be biologically explained though the competition between both species likely increases cell mortality, which may reduce the maximum growth rate of *W. anomalus*. However, the release of amino acids associated with this cell death could contribute to an increase in carrying capacity by enhancing organic nitrogen availability (Jones, 1999; Toro & Vazquez, 2002). Furthermore, the commonly assumed positive relationship between equilibrium population density and competitive ability is often invalid (Fronhofer *et al.*, 2024).

3. Time integral evaluation – *Wickerhamomyces anomalus* presence

The kinetic profiles showed better establishment and persistence of *W. anomalus* when co-inoculation was performed at 48 h, followed by 24 h. On average, co-inoculation at 48 h resulted in the highest growth and persistence of the biocontrol yeast; taking this condition as 100 %, co-inoculation at 24 h reduced its population approximately by 35.3 % and simultaneous inoculation (0 h) by 75 %.

Maintaining a high *W. anomalus* population over time is crucial for effective biocontrol against spoilage yeasts (Canonico *et al.*, 2023a; Canonico *et al.*, 2023b). However, this behaviour is likely not a constant behaviour, as the growth kinetics of *W. anomalus* tend to decline around the fourth day, probably due to the decrease in dissolved oxygen (~80 %) (Pérez-Magariño *et al.*, 2023), which limits its aerobic metabolism (Mehlomakulu *et al.*, 2021).

The impact of *W. anomalus* co-inoculation on *S. cerevisiae* was minimal, consistent with Gallo *et al.* (2024), who also found no relevant effects of *Hanseniaspora vinae* on the fermentative yeast. Nevertheless, Onetto *et al.*, (2024) reported a significant interference of *Hanseniaspora uvarum* with *S. cerevisiae*, suggesting that the magnitude of the interaction depends on the specific non-*Saccharomyces* species involved and on the medium conditions.

4. Simulation and validation of different inoculum scenarios

Figure 4 illustrates the population dynamics across different initial inoculum concentrations and co-inoculation times: 0 h (Figure 4A), 24 h (Figure 4B), and 48 h (Figure 4C). This analysis is conceptually similar to the nullcline analysis (Zeeman, 1995). It evaluates the temporal evolution of both populations across 400 simulations, enabling visualisation of their overall trends.

The experimental validations (Figure 5), performed with high initial concentrations of *W. anomalus* and low concentrations of *S. cerevisiae*, confirmed the model simulations: in all three co-inoculation times, the fermenting yeast reached a carrying capacity close to 7.64 $\log_{10}$ cells/mL (4.7×10^7 cells/mL), converging to the same final sink state. This indicates that the order of arrival, or the population size of the colonising organism, did not negatively affect the competitive outcome, deviating from an inhibitory priority effect and instead suggesting a neutral or even facilitative effect (Stroud *et al.*, 2024). Unlike natural systems described by Toju *et al.* (2018), where early-arriving species modified the niche and inhibited subsequent ones, here the anthropic introduction of *S. cerevisiae* imposed a predictable competitive direction.

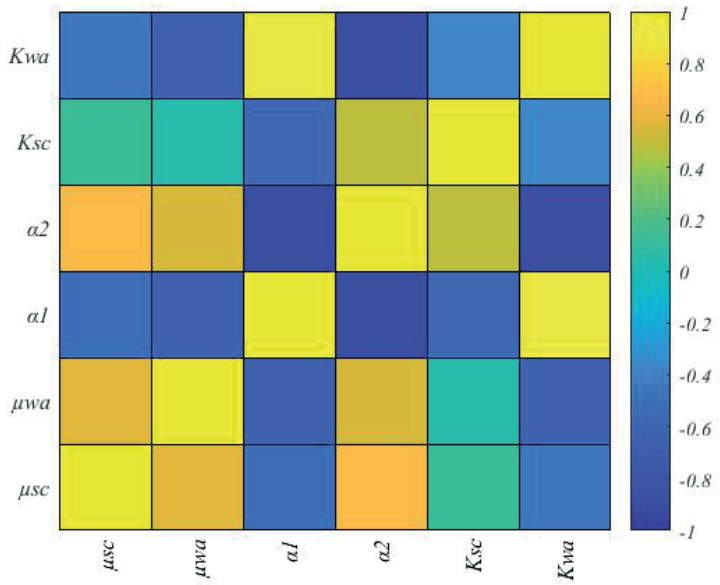


FIGURE 3. Crammer–Rao matrix for global unknowns: parametric correlation analysis of the complete Lotka–Volterra model.

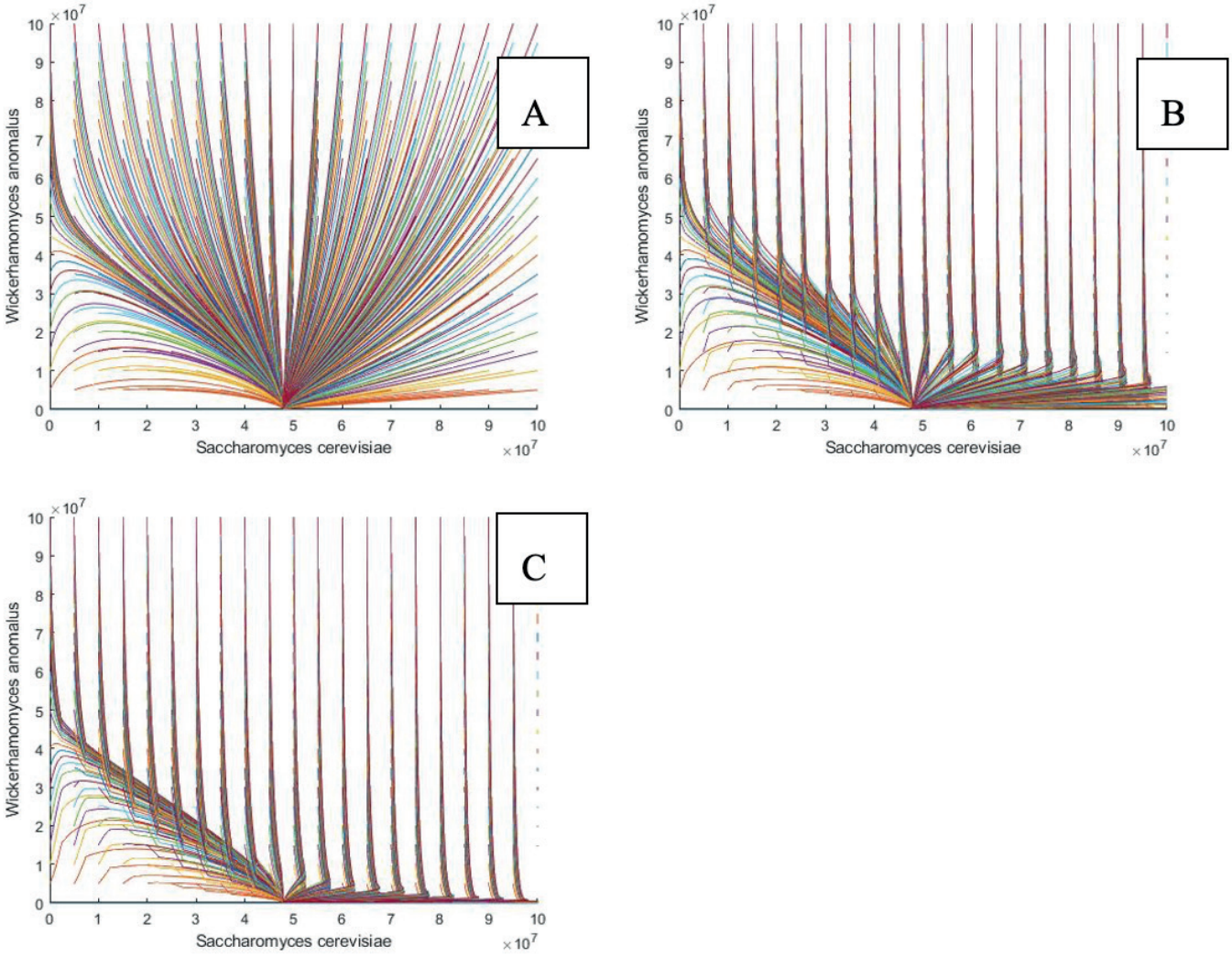


FIGURE 4. Population dynamics under different co-inoculation initial proportions at different co-inoculation times. A: 0 h, B: 24 h, C: 48 h.

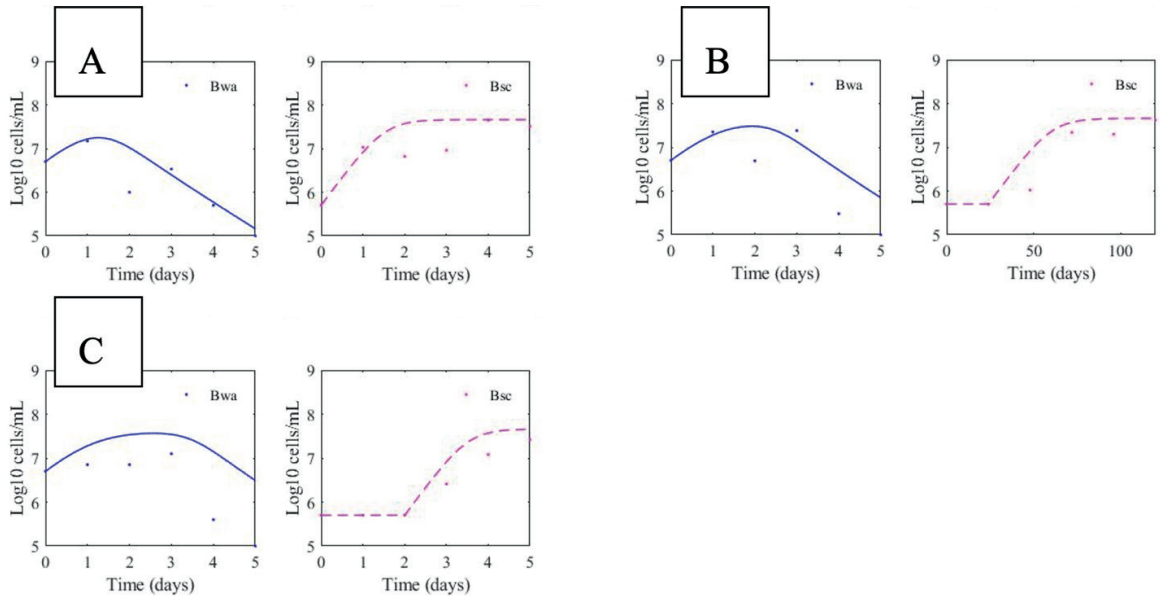


FIGURE 5. Validation: different co-inoculation initial proportions, *W. anomalus* 5 × 10^6 cells/mL and *S. cerevisiae* 5 × 10^5 cells/mL, at different co-inoculation times. A: 0 h, B: 24 h, C: 48 h.

CONCLUSION

The modified Lotka–Volterra model was successfully fitted and validated to describe the dynamics of sequential mixed co-inoculation between the non-conventional yeast and *S. cerevisiae*, considering different inoculation times and initial population sizes.

Within the evaluated experimental range, temperature and SO_2 concentration showed no significant effects on population dynamics, whereas co-inoculation timing was identified as the only determining factor.

Delaying the addition of *S. cerevisiae* transiently favoured the establishment of *W. anomalus*; however, the system rapidly shifted toward the competitive dominance of the fermentative yeast.

The order of arrival did not affect the final competitive outcome or sink state, contrary to expectations for an inhibitory priority effect. Even at low initial proportions, *S. cerevisiae* consistently prevailed in the experimental validations, reaching the same equilibrium point predicted by its carrying capacity (4.7 × 10^7 cells/mL).

The greater persistence of *W. anomalus* and its lack of interference with *S. cerevisiae* under the conditions outlined in this study highlight its potential as a biocontrol agent, representing a promising strategy to reduce SO_2 use and promote more sustainable winemaking practices.

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DECLARATION OF CONFLICTING INTERESTS

The authors declare that they have no known financial conflicts of interest or personal relationships that could have influenced the work presented in this article.

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