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**PROBIOTIC CAPABILITY IN YEASTS: SET-UP OF A SCREENING METHOD**

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**ABSTRACT**

Microbial probiotic supplements are used to improve the health and wellness of people. For checking this ability (resistance to gastrointestinal conditions, auto-aggregation, cell surface hydrophobicity or biofilm formation abilities), it is crucial to evaluate some kinetics parameters. The objective of the present study was to establish an adequate method for studying the probiotic potential of *Saccharomyces* and non-*Saccharomyces* strains isolated from food ecosystems. A new protocol was designed based on kinetics parameters: lag phase ( $\lambda$ ), generation time (G) and maximum OD (OD<sub>max</sub>). The design was done using a step-by-step approach (up to 10 protocols), focusing on the choice of controls and the conditions to simulate the stomach and intestine. Each decision was made based on multifactorial statistical assay results. A preliminary screening indicates that protocol is adequate, quick and reproducible and that *S. cerevisiae* strains are more resistant than non-*Saccharomyces* ones. *S. boulardii*, (commercial control), was not one of the best yeasts evaluated. Thus, the most relevant results of the work are the setup of a new method for studying the survival/resistance of yeasts to the transit into the gut based on a complex statistical analysis of results and to mark the probiotic character in *Saccharomyces* yeasts versus non-*Saccharomyces*.

**Highlights**

New method for studying the survival/resistance of yeasts during the transit into the gut.

Statistical analysis of growth kinetic parameters of probiotic yeasts.

Marked probiotic character in *Saccharomyces* yeasts versus non-*Saccharomyces*.

**Keywords**

Probiotic yeasts, new method, kinetic parameters, statistical analysis.

## 1. Introduction

Gastrointestinal microbiota plays an important role in waterborne diseases, constituting an extremely complex ecosystem. When this balance is altered, a series of clinical disorders is triggered. Recent studies have linked the imbalance of gut microbiota with gastrointestinal disorders (Czerucka, Piche, & Rampal, 2007). In addition, the microbiota has been proposed as an important regulator of the immune system outside of the intestine. Attempts have been made to improve the health of people affected by gut microbial modulation using a microbial probiotic supplement must be able to grow and persist in the human intestine (Rivera-Espinoza & Gallardo-Navarro, 2010).

There is increasing evidence related to the use of probiotics in functional foods, dairy products and other diet supplements used for maintaining and promoting human health (Jankovic, Sybesma, Phothirat, Ananta, & Mercenier, 2010). Functional foods normally have lactic bacteria strains, other bacteria or yeasts (Wassenaar & Klein, 2008). Although the role of yeasts is well documented in the development of new foods as well as in their important role in the fermentation process, the characterization of their probiotic activities is very limited (Kumura, Tanoue, Tsukahara, Tanaka, & Shimazaki, 2004, Romanin et al., 2010).

For this reason, it is necessary to go deeply in the study of yeasts as probiotics, for verifying their interesting properties. Their size is 10 times bigger than bacteria, which is an important steric hindrance against them (Czerucka et al., 2007). However, they are normally resistant to antibiotics which requires maintaining in-gut biota during antibiotic treatments (Czerucka et al., 2007). They cannot spread the genes of antibiotic resistance and their translocation has never been reported. On the other hand, they are sometimes resistant to the transit into the gut, produce vitamins in situ and can reduce the content of mycotoxins in food and in the gut.

Recent researches show that some of the species with probiotic evidence are *Saccharomyces cerevisiae* var. *boulardii*, *Kluyveromyces marxianus*, and *Pichia kudriavzeii*. The EFSA has awarded the QPS status to only a few yeasts as “food additive”, i.e. *Kl. marxianus* var. *lactis* and *Kl. marxianus fragilis*. Nevertheless, there is only one patented yeast strain that has been efficient in double blind studies (Sazawal, Hiremath, & Dhingra, 2006): *Saccharomyces cerevisiae* var. *boulardii*. This yeast is commercialized in many countries as a probiotic because it survives the passage through the intestinal tract, presents good growth at 37°C and inhibits pathogen growth. Therefore, it is used as a preventive and therapeutic agent against diarrheal and other gastrointestinal disorders caused by antibiotic administrations.

On the other hand, gut microbiota varies with regard to number and species depending on ambient conditions such pH, presence of enzymes, of bile salts and pancreatic fluids (Berg, 1996, Appleby & Walters, 2014).

Yeasts form less than 0.1% of the intestinal gut residue. Yeasts are present in the stomach and the colon due to their resistance at pH levels. While their optimum pH is between 4.5 and 6.5, the majority of them can grow in the pH range of 3-8 and some of them can resist more acidic conditions, up to pH 1.5. This is a good starting point for considering yeasts as probiotics, although they must also resist the presence of gastrointestinal enzymes, bile salts, organic acids and changes in temperature (Czerucka et al., 2007). The majority of yeasts isolated from the gut are *Candida albicans*, although on a few occasions *Torulopsis glabrata* and *Candida tropicalis* have also been isolated (Giuliano, Barza, & Jacobus, 1987).

The consumer market of probiotic foods amounted to 1.4 billion euros, with an estimated annual growth of between 7% and 8 %, for the period 2008 to 2013 (Saxelin, 2008), and in particular, 20% of fermented dairy products contained probiotics (Wassenaar & Klein, 2008).

For this reason, research in this field has also increased in recent years. There are some studies which evaluate the probiotic characteristics in yeasts (Kumura et al., 2004; Pedersen, Owusu-Kwateng, Thorsen, & Jespersen, 2012; Diosma, Romanin, Rey-

Burusco, Londero, & Garrote, 2014; Gil-Rodriguez, Carrascosa, & Requena, 2015) but none of them is totally adequate. For example, Kumura et al. (2004) do not evaluate the presence of enzymes in the gut and Pedersen et al (2012), do not differentiate between the steps of the process (stomach and intestine). On the other hand, all of them study the viable cells after assays but the yeast kinetic is not followed and so there is no data about important parameters such as lag phase, generation time and maximum specific growth rate.

Due to the interest of the food industry in the selection of new probiotic strains, the objective of the present study was to establish an adequate method for evaluating the probiotic potential of microorganisms and to use it for the evaluation of *Saccharomyces* and non *Saccharomyces* yeasts isolated from wild food ecosystems.

Therefore, the main goal of this paper was to design a new laboratory protocol to study the survival/resistance of yeasts to the transit into the gut. This main goal has been addressed by some intermediary milestones:

- a) The choice of positive and negative controls
- b) The design of the conditions to simulate the gut
- c) A validation with some other yeasts

## **2. Materials and methods**

### **2.1. Yeast strains**

The yeast strains (*Saccharomyces* and non-*Saccharomyces* genera) used belong to the culture collection of the Yeast Biotechnology Laboratory of the University of Castilla-La Mancha and to the “Colección Española de Cultivos Tipo” (CECT). There was also one commercial strain, *Saccharomyces cerevisiae boulardii*, used as a control due to its probiotic properties (Table 1). Its commercial name in the pharmacy in Spain, is *ultralevura*<sup>®</sup> and it is recommended for recovering the balance of the gastrointestinal microbiota. All yeasts from the laboratory were isolated from their natural environment and, together with the rest, were kept in 15% glycerol at – 80°C.

For the setting up of the method, three *Saccharomyces cerevisiae* (labelled 2, 3 and 4) were used, the commercial strain (24) and three non-*Saccharomyces* yeasts (*Debaryomyces hansenii* (1001), *Kluyveromyces lactis* (1121) and *Torulaspora delbrueckii* (1567), (Table 1A).

To test the method obtained to determine the resistance to passage through the gastrointestinal tract (application) all strains used for setting up were used again, as well as five *Saccharomyces cerevisiae* from the UCLM laboratory (1, 7, 39, 111 and 142) and four non-*Saccharomyces* yeasts: three from UCLM (*Rhodotorula mucilaginosa*, 1017; *Zygosaccharomyces fermentati*, 1061; and *Candida vini*, 1063) and one from CECT (*Hanseniaspora uvarum*, 1511) (Table 1B).

## 2.2. Setting up of the method

Set-up of the method was carried out bearing in mind the following objectives:

- Selection of positive and negative controls.
- Establishment of the best conditions for simulating intestinal gut digestion to obtain a rapid, reproducible and reliable screening method.

The set-up method was a sequential process in which the control microorganisms and the most adequate conditions were selected systematically. Each step of the process is detailed in Table 2.

To carry out the set-up, 10 different protocols were designed, each with at least two consecutive steps (static phase and growth kinetic phase) (Table 2). The strains shown in Table 1A were used. A colony of fresh culture of each yeast was picked and inoculated in 4 ml of YPD broth (1% yeast extract, 2% peptone and 2% glucose) and incubated with agitation at 30 °C for 24 h. Then, cells were separated by centrifugation at 5000 rpm for 5 min, and pellets were washed twice in a sterile phosphate-buffered saline (PBS) pH 7.2 for inoculating with a concentration of  $10^8$  cfu/mL at 37°C without agitation under different assay conditions (media, pH, and time) (see Table 2 “static phase”).

After the static phase, the growth of yeasts was monitored at 600 nm in an Absorbance Microplate Reader ELx808 (Bio-Tek Instruments, Vermont, USA) under

different conditions (growth media, pH, time and temperature) (see Table 2 “growth kinetic phase”). The wells of the microplate were filled with 0.2 mL of different studied media inoculated in the corresponding static phase with  $10^6$  cfu/mL. Measurements were taken every 20 minutes for 24 hours, with an agitation period of 15 s before reading.

Growth curves were obtained by plotting optical density (OD) at 600 nm versus time (t). The most important parameters calculated using the model described by Warringer & Blomberg (2003) were: lag phase (L), generation time (G), and maximum OD (OD<sub>max</sub>) reach at stationary phase. The specific growth rate constant ( $\mu_{max}$ ) was calculated ( $\mu_{max} = \log 2 / G$ , where G stands for generation time).

For selecting the positive and negative controls, the work was done with protocols 1 to 5 and strains 2, 3, 4, 24, 1001, 1121, 1567. To establish the best conditions for simulating intestinal gut digestion, protocols from 6 to 10 were used, applied on strains 3 and 24.

The viable count before and after the protocols was assessed by seeding on YPD agar plates (30 °C/48 h)

### **2.3. Application**

Resistance to passage through the gastrointestinal tract of the strain collected in Table 1B was studied. It was useful to know if the protocol obtained was valid or not.

Cells were refreshed on YPD agar and after 48 h of incubation at 30°C, colonies were picked and directly used to inoculate 4 ml of fresh YPD broth. These tubes were then incubated with agitation at 30°C for 24 h. The cells were then separated by centrifugation at 5000 rpm for 5 min, the pellets were washed twice in a sterile phosphate-buffered saline (PBS) pH 7.2, and re-suspended for development of the method set up in the previous stage.

In both set-up and application stages, assays were performed in four-fold.



## **2.4. Statistical analyses**

Statistical Analysis was performed through the software Statistica for Windows ver. 12.0 (Statsoft, Tulsa, Okla.) and IBM SPSS for Windows ver. 22.

### **2.4.1. Setting up**

#### **2.4.1.1. Selection of controls**

To select the positive and negative controls, the results from protocols 1- 3 were first analyzed through a multifactorial ANOVA. The kind of protocol (1, 2, and 3) and the yeasts (2, 3, 4, 24, 1001, 1121, 1567) were used as categorical predictors, whereas the fitting parameters of the equation of Warringer & Blomberg (2003) ( $\lambda$ , G,  $OD_{max}$ ), and the specific growth rate ( $\mu_{max}$ ) were set as dependent variables.

The main outputs of this approach were the tables of standardized effects and the decomposition of statistical hypothesis (figures). A table of standardized effects shows the Fisher-test, and the significance of the individual (protocol and yeast) and interactive effects of predictors (protocol\*yeast). The graphs of the decomposition of statistical hypothesis report on the correlation of each predictor versus the dependent variables.

As a final step, the global differences amongst the three protocols were analyzed through a Principal Component Analysis; the mean values of the fitting parameters of Warringer & Blomberg (2003), and the specific growth rate from each yeast and each protocol were used as input variables.

After that, the data from protocols 4 and 5 was analyzed as variability plots. The strains were used as categorical predictors, whereas the fitting parameters were set as dependent variables. A variability plot shows all replicates of the dependent variables in a box and the mean/median value as a line.

A two-way ANOVA was used to analyze the results. The kind of protocols/yeasts and the fitting parameters of Warringer & Blomberg (2003) were respectively set as categorical predictors and dependent variables.

#### **2.4.1.2. Selection of conditions for simulating intestinal gut digestion**

Finally, to select the best conditions for simulating intestinal gut digestion, protocols 6 to 10 were tested. The global differences were analyzed through a Principal Component Analysis using the parameters of strains 3 and 24.

#### **2.4.2 Application**

To check if the protocol obtained was valid or not, a one-way ANOVA was used for comparing the data obtained during the evaluation of resistance to passage through the gastrointestinal tract.

### **3. Results and discussion**

#### **3.1. Setting up**

##### **3.1.1. Selection of controls**

The results from protocols 1-3 were analyzed through a two-way ANOVA to pinpoint the strains to be used as positive and negative controls. Table 3 shows the standardized effects and the significance of the categorical predictors (yeast and protocol) and their interaction. The predictor “protocol” never played a significant role as a single term, whilst the predictor “yeast” was always significant. The interaction “yeast\*protocol” was significant for the lag phase and the maximum OD.

A second output of two-way ANOVA is the decomposition of the statistical hypothesis, which could give a quantitative output on the correlation predictors/dependent variables. The decomposition is shown in Figure 1.

Strains 2, 4 and 1567 were not able to grow under the conditions specified (strains 2 and 4 were not used in the graph since their values were the same as 1567-no growth-).

The specific growth rate was maximum for strain 3 (*S. cerevisiae*), and minimum for strains 1001 and 1567 respectively (*D. hansenii* and *T. delbrueckii*) (Figure 1A) Strains 24 (*S. cerevisiae* var. *boulardii*) and 1121 (*K. lactis*) experienced an intermediate trend. The generation time was minimum and in the range 2-3 h for strains 3, 24, and 1121 and maximum (ca. 22 h) for strain 1567 (no growth) (Figure 1B). Concerning the lag phase, the statistic did not find significant differences between strains 3, 24, 1001 and

1121 and found the maximum value for strain 1567 (Figure 1C). Finally, the  $OD_{max}$ , which is an indirect measure of the value of biomass in the steady state, was maximum for strain 3 (Figure 1D).

Ideally, a positive control should have the minimum value of the lag phase and generation time, and the highest levels for the OD and the specific growth rate. On the other hand, a negative control should not grow in the tested conditions. The decomposition of the statistical hypothesis offers some interesting outputs for each strain and suggests that strains 3 and 1567 could be promising as positive and negative controls, respectively.

However, each parameter was analyzed separately, therefore a final PCA was run to analyze the global differences of the target strains (Figure 2). The projection factor 1 x factor 2 accounted for ca. 95% of the total variability; the input variables (lag phase, generation time, specific growth rate and  $OD_{max}$ ) were related to factor 1. The correlation OD/specific growth rate versus factor 1 was positive, but was negative for the other variables (Figure 2A).

The projection of the cases (Figure 2B) confirmed the suitability of strain 1567 as a negative control. It was placed in the quadrant III (maximum values for the lag phase and the generation time and minimum values for OD and growth rate). The best performances in terms of growth kinetic were found for strain 3 (quadrant IV) (positive control). Strains 24 and 1121 showed a promising trend, although their performances were lower than those reported for the positive control.

Next, the growth kinetic of strains was assessed after the exposure to conditions simulating the stomach (protocol 4) and the intestine (protocol 5) (Figure 3). The exposure to pepsin (stomach-protocol 4) exerted a strong inhibition on the target strains as they did not grow after cell harvesting and inoculation in fresh YPD (high values of lag and generation time, low value of specific growth rate), with some exceptions identified in some replicates of the strain 1121. The exposure to pancreatin and bile salt exerted a weaker effect, as many strains experienced a growth kinetic; moreover, strain 24 showed better performances than *S. cerevisiae* strain 3. Strain

1121 was less resistant to intestinal than in the gastric conditions and suffered a significant inhibition.

The viable count was always assessed after the exposure to stomach and intestinal conditions; the effect of gastric and intestinal conditions was always slight, as yeast count decreased by 1 log cfu/ml or less.

Combining the outputs from protocols 1-3 (two-way ANOVA and PCA) and protocols 4 and 5 (variability plot), the positive and negative controls could be chosen.

As positive controls, strains 3, *S. cerevisiae* (best performances in the protocols 1-3), and 24, *S. boulardii* (better performances after exposure to pancreatin and bile salts), were chosen. Moreover, it was important to consider strain 24 due to it being a commercial yeast strain used as a probiotic. As a negative control, strain 1567, *T. delbrueckii*, was considered the most adequate.

Concerning the pH of the stomach, a value of 2 was chosen because the differences between protocol 1 (pH 2.5 for the stomach) and 2 (pH 2 for the stomach) were not significant.

### **3.1.2. Selection of conditions for simulating intestinal gut digestion**

The second step of the set-up was to design the best conditions to simulate the transit into the gut and propose a protocol to select yeasts capable of survival. Protocols 6 and 7 included the exposure to pepsin (step A) and pancreatin/ bile salts (step B), cell harvesting and inoculation in a fresh medium. On the other hand, in protocols 8-10, pancreatin and bile salts were added to the medium used for the growth kinetic. For these protocols, the differences relied upon the kind of medium. This is an important factor since to these variables affect to the viability of yeasts as other authors reported (Psomas et al., 2001, Kumura et al., 2004; Kühle et al., 2005, Pedersen et al., 2012; Diosma et al., 2014; Gil-Rodriguez et al., 2015).

Strains 3 and 24 (positive controls) were always able to grow, thus they were selected as target strains to run a two-way ANOVA and compare the performances of the different protocols. Protocol 6 was excluded due to the high variability in the results.

The standardized effects are shown in Table 4. The predictor "protocol" was always significant; the predictor yeast was significant for the specific growth rate and the OD,

but its significance was lower than the “protocol”. The decomposition of the statistical hypothesis for the protocol can be seen in Figure 4.

The specific growth rate and the OD were maximum for protocol 10 and minimum in protocol 7; the generation time was maximum in protocol 8, whereas protocols 7, 9, and 10 showed similar performances. As a final step, a PCA was run to compare the global differences (Figure 5). The best performances (high specific growth rate and OD and low generation time) were found for both strains 3 and 24 after exposure to protocol 10. Thus, protocol 10 was chosen as the best compromise and was proposed as a possible tool to study and select promising probiotic yeasts.

All results complete the information that there already is in the scientific community. Some studies (Kumura et al., 2004; Pedersen et al., 2012; Diosma et al., 2014; Gil-Rodriguez et al., 2015) evaluate the probiotic characteristics in yeasts but none of them is so rigorous in the assays since they do not difference between the steps of the process (stomach and intestine).

Another approach of this study is the kinetic growth obtained, since all studies only offer the number of viable cells after assays and there is not information about some important parameters such as lag phase, rate or maximum growth.

### **3.2. Application**

Table 5 shows the fitting parameters of all the strains after exposure to protocol 10. A one-way ANOVA was used for comparing the data obtained during the evaluation of resistance to passage through the gastrointestinal tract.

In general, it is shown that gastrointestinal transit conditions affect yeast viability (56% of evaluated yeasts were not able to grow before 22h under these conditions). Others authors show similar results: Diosma et al. (2014) obtained a decrease of viability in more than 50% of cases in similar situation; only the 44% of evaluated yeasts by Kühle et al. (2015) were able to grow at pH 2,5 and Psomas et al. (2001) showed that some *S. cerevisiae*, *T. delbrueckii* and *P.farinosa* strains did not grow at pH values less than 3. Nevertheless, Pedersen et al. (2012) found that all strains studied were able to grow under GI transit conditions although with different rate.

For  $\mu_{\max}$ , four significantly different groups were stabilized (a to d). Yeasts in group “a” (2, 4, 142, 1001, 1017, 1061, 1121, 1511, 1567) were not detected, therefore they are not able to resist the probiotic conditions. They were the three *S. cerevisiae* (2, 4, and 142) and six non-*Saccharomyces* yeasts (*D. hansenii*, *R. mucilaginosa*, *Z. fermentati*, *K. lactis*, *H. uvarum* and the negative control, *T. delbrueckii*). In group “b” there was the positive control *S. boulardii* (24) and one non-*Saccharomyces* (1063, *Candida vini*). followed by group “c” with three *S. cerevisiae* (1, 111 and the other positive control, 3), and the strains 7 and 39, *S. cerevisiae* in group “d” with the highest values (0,21 and 0,205 h<sup>-1</sup> respectively).

The results of the present study show that the positive control *S. boulardii* (24), does not present the maximum rate growth which agree with the result obtained by others authors; Diosma et al., (2014) found that *S. boulardii* was more sensitive to bile salts that other wild strains.

On the other hand, Gil Rodriguez et al (2015) found  $\mu_{\max}$  values lower than the ones obtained in the present study (0,06 to 0,12 versus 0,11 to 0,21 respectively).

The statistics test in the variable generation time (G), divided the yeasts into five different groups. As before, in the first one there were those that not were able to grow (group “a”) and in the second one, the yeasts 24 and 1063 (group “b”). The next one was the positive control 3, unique in group “c”, followed by 1, 7 and 111 (“d”) and in group “e”, yeast 39 was the best one with the shortest time generation (1.48 h).

Finally, for maximum OD, the ANOVA also stabilized five different groups with 1063 (group “b”) being the worst one after the no growth ones (group “a”), followed by 24 (“c”), 7 and 111 (groups “c” and “d”) and strain 39 was again the best one in group “e” with a value of 1.47.

As an example of the kinetic curves obtained, Figure 6 shows the graph for the positive and negative controls (3 and 1567), *S. boulardii* (24), and the yeast with the best results (39).

#### 4. Conclusions

The design of laboratory protocol is a complex process, involving the choice of lab conditions, the use of statistics, and validation. In this paper, the design was done using a step-by-step approach, focusing on the positive and negative controls, on the choice of the conditions to simulate the stomach and intestine, and a final validation.

As positive and negative controls, *S. cerevisiae* strain 3 and *T. delbrueckii* strain 1567 were chosen because of their performances and growth kinetic. In addition, strain 24 was also taken as a control due to it being a commercial probiotic yeast.

The simulation conditions of the stomach and intestine were based on the following protocol: a first step with static conditions for 3 h. at pH 2 and 37°C in the presence of pepsin; and a second step which was a growth assay in the presence of bile salts and pancreatin for 22 h. at pH 8 and 37°C to simulate the intestine (protocol 10).

Protocols in which the time of the process and the media composition were closer to the real conditions (protocols 6, 7, 8 and 9) showed the same trend as protocol 10, so they were discarded as a screening method, resulting in a significant saving in time and money.

Apart from the design of the protocol, this paper offers some other interesting evidence.

The preliminary screening indicates that *S.cerevisiae* strains are more resistant to the enteric conditions than non-*Saccharomyces* ones, which seems to indicate that the probiotic character of *Saccharomcyes* yeasts is more accentuated.

Surprisingly, yeast 24, *S.boulardii*, used as a positive control due to commercial characteristics, is not one of the best yeasts evaluated.

There was only one non-*Saccharomyces* strain, 1063 *C. vini*, that resisted the probiotic conditions, although its values always presented an intermediate trend, being close to data from yeast 24.

The best yeast evaluated was number 39, one *S. cerevisiae* isolated from a wine environment and belonging to the culture collection of the UCLM laboratory.

The results obtained in the present study showed that the protocol set-up is adequate and it can be useful for evaluating the probiotic characteristics and studying the growth kinetic along the sequential process throughout the intestinal gut while being, on the other hand, a quick and reproducible method.

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**Figure captions**

**Figure 1:** Decomposition of the statistical hypothesis for the effect of the predictor “yeast” in the protocols 1-3. Vertical bars denote 95% confidence intervals.

**Figure 2:** Principal Component Analysis run on the fitting parameters of Warringer and Blomberg (2003) recovered for the strains 3, 24, 1001, 1121, and 1567 in the protocols 1, 2, and 3. The analysis was plotted on a two-dimensional space by two axis (factors). The factor 1 accounted for the lag phase (lag), the generation time (G), the growth rate (rate) and the maximum OD (OD).

A), variable projection (correlation of the variables with the statistical components); B), case projection (yeast distribution as a function of the variables).

**Figure 3:** Variability plots for the parameters of Warringer and Blomberg (2003) recovered for the strains 3, 24, 1001, 1121, and 1567 in the protocols 4, and 5. Each bar was coded as “strain-protocol”, i.e. the first number is for the strain and the second for the protocol.

**Figure 4:** Decomposition of the statistical hypothesis for the effect of the predictor “protocol” in the protocols 7-10. Vertical bars denote 95% confidence intervals.

**Figure 5:** Principal Component Analysis run on the fitting parameters of Warringer and Blomberg (2003) recovered for the strains 3, and 24, in the protocols 7-10. The analysis was plotted on a two-dimensional space by two axis (factors). The factor 1 accounted for the lag phase (lag), the generation time (G) and the maximum OD (OD).

A), variable projection (correlation of the variables with the statistical components); B), case projection (yeast distribution as a function of the variables).

**Figure 6:** Kinetic growth for strain 3, positive control (A), 1567, negative control (B), 24, *Saccharomyces boulardii* (C), 39 (D).

Table 1A. Yeast strains from culture collection of the Yeast Biotechnology Laboratory of the University of Castilla-La Mancha and to the “Colección Española de Cultivos Tipo” (CECT) used for the setting up of the method.

YEAST NUMBER	YEAST SPECIES	SOURCE
2	<i>Saccharomyces cerevisiae</i>	UCLM
3	<i>S. cerevisiae</i>	UCLM
4	<i>S. cerevisiae</i>	UCLM
24	<i>S. cerevisiae boulardii</i>	Commercial yeast
1001	<i>Debaryomyces hansenii</i>	UCLM laboratory
1121	<i>Kluyveromyces lactis</i>	UCLM
1567	<i>Torulaspora delbrueckii</i>	CECT 1880

Tabla 1B. Yeast strains used for the application of the method

YEAST NUMBER	YEAST SPECIES	SOURCE
1	<i>Saccharomyces cerevisiae</i>	UCLM
2	<i>S. cerevisiae</i>	UCLM
3	<i>S. cerevisiae</i>	UCLM
4	<i>S. cerevisiae</i>	UCLM
7	<i>S. cerevisiae</i>	UCLM
39	<i>S. cerevisiae</i>	UCLM
111	<i>S. cerevisiae</i>	UCLM
142	<i>S. cerevisiae</i>	UCLM
24	<i>S. cerevisiae boulardii</i>	Commercial yeast
1001	<i>Debaryomyces hansenii</i>	UCLM
1017	<i>Rhodotorula mucilaginosa</i>	UCLM
1061	<i>Zygosaccharomyces fermentati</i>	UCLM
1063	<i>Candida vini</i>	UCLM
1121	<i>Kluyveromyces lactis</i>	UCLM
1511	<i>Hanseniaspora uvarum</i>	CECT 11106
1567	<i>Torulaspora delbrueckii</i>	CECT 1880



**Table 3:** Two way ANOVA for the effects of the kind of approach (protocols 1, 2 or 3) and yeasts used to develop a protocol to simulate the transit into the gut. The two input variables were coded as “protocol” and “yeast”. F, Fisher test value; SS, sum of residual square. MS, mean square residual; df, degree of freedom.

Predictors	SS	df	MS	F
<b><math>\mu_{\max}</math></b>				
Protocol	-*	-	-	-
Yeast	0.20	4	0.05	104.34
Protocol*Yeast	-	-	-	-
<b>G</b>				
Protocol	-	-	-	-
Yeast	8.15	2	4.07	1.09
Protocol*Yeast	-	-	-	-
<b><math>\lambda</math></b>				
Protocol	-	-	-	-
Yeast	2114.375	4	528.59	926.69
Protocol*Yeast	33.09	8	4.14	7.25
<b><math>OD_{\max}</math></b>				
Protocol	-	-	-	-
Yeast	13.46	4	3.37	68.27
Protocol*Yeast	1.31	8	0.16	3.33

\*Not significant

$\mu_{\max}$ , specific growth rate; G, generation time;  $\lambda$ , lag phase;  $OD_{\max}$ , maximum OD.

**Table 4:** Two way ANOVA for the effects of the kind of approach (protocols 7, 8, 9 or 10) and yeasts used to develop a protocol to simulate the transit into the gut. The two input variables were coded as “protocol” and “yeast”. F, Fisher test value; SS, sum of residual square. MS, mean square residual; df, degree of freedom.

Predictors	SS	df	MS	F
<b><math>\mu_{\max}</math></b>				
Protocol	0.05	3	0.02	32.78
Yeast	0.00	1	0.00	4.99
Protocol*Yeast	-*	-	-	-
<b>G</b>				
Protocol	134869.52	3	44956.51	6.76
Yeast	-	-	-	-
Protocol*Yeast	-	-	-	-
<b><math>OD_{\max}</math></b>				
Protocol	2.08	3	0.69	19.53
Yeast	0.35	1	0.35	9.74
Protocol*Yeast	0.52	3	0.17	4.87

\*Not significant

$\mu_{\max}$ , specific growth rate; G, generation time;  $\lambda$ , lag phase;  $OD_{\max}$ , maximum OD.

Table 2. Protocols applied for setting up the method for studying the behaviour of yeast along the gut passage.

Protocol number	Static phase (1) ( $10^8$ cfu/mL, 37°C, no agitation)			Growth kinetic phase (2) ( $10^6$ cfu/mL)				Total time T (h)
	Media	pH	T (h)	Media	pH	T (h)	T <sup>a</sup> (°C)	
1	PBS	2	3	YPD	6,5	20	30	23
2		2,5						23
3		8	4					24
4	PBS + pepsine (3 mg/mL)	2	3			22	37	25
5	PBS + pancreatic enzymes (1 mg/mL) + biliary salts (0,5%)	8	4					26
6*	A. PBS + pepsin (3 mg/mL)	2	3			-	-	-
	B. PBS + pancreatic enzymes (1 mg/mL) + bile salts (0,5%)	8	4	YPD	8	22	37	
7*	A. PBS + pepsin (3 mg/mL)	2	3	-	-	-	-	29
	B. YNB + pancreatic enzymes (1 mg/mL) + bile salts (0,5%)	8	4	YPD	8	22	37	
8	PBS + pepsin (3 mg/mL)	2	3	YNB + glucose (0,5 %) + pancreatic enzymes (1 mg/mL) + bile salts (0,5%)		72		75
9				YM + pancreatic enzymes (1 mg/mL) + bile salts (0,5%)		22		25
10				YPD + pancreatic enzymes (1 mg/mL) + bile salts (0,5%)	22	25		

\*In step 6 and 7, assay A is followed to assay B by centrifugation and washed of pellet with the medium of assay B. After assay B, growth kinetic is studied under specified conditions. YPD: yeast extract peptone dextrose, YNB: yeast nitrogen base, YM: yeast medium.

**Table 5:** Fitting parameters of Warringer and Blomberg (2003) of the target yeasts in the protocol 10. Mean values $\pm$ standard deviation.

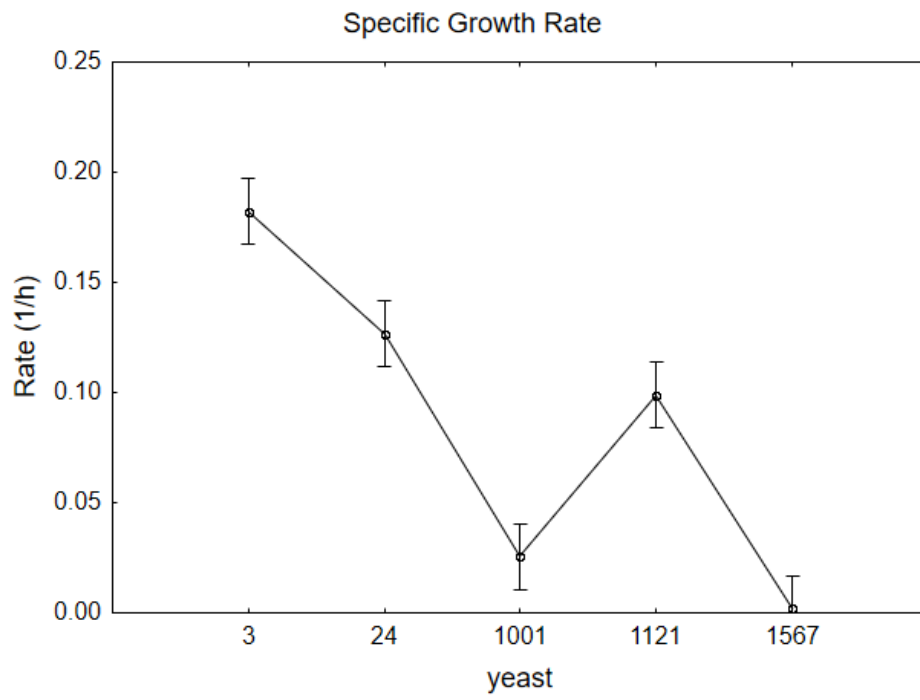
Strains	$\mu_{\max}$ (1/h)	G (h)	OD <sub>max</sub>
1	0.15 $\pm$ 0.01 <sup>c</sup>	2.01 $\pm$ 0.07 <sup>d</sup>	1.33 $\pm$ 0.03 <sup>d</sup>
2	nd <sup>a</sup>	> 22.00 <sup>a</sup>	nd <sup>a</sup>
3	0.15 $\pm$ 0.01 <sup>c</sup>	2.36 $\pm$ 0.09 <sup>c</sup>	1.30 $\pm$ 0.03 <sup>d</sup>
4	nd <sup>a</sup>	> 22.00 <sup>a</sup>	nd <sup>a</sup>
7	0.21 $\pm$ 0.02 <sup>d</sup>	2.01 $\pm$ 0.20 <sup>d</sup>	1.22 $\pm$ 0.01 <sup>c,d</sup>
24	0.11 $\pm$ 0.00 <sup>b</sup>	2.75 $\pm$ 0.41 <sup>b</sup>	1.21 $\pm$ 0.02 <sup>c</sup>
39	0,205 $\pm$ 0.02 <sup>d</sup>	1.48 $\pm$ 0.13 <sup>e</sup>	1.47 $\pm$ 0.09 <sup>e</sup>
111	0.14 $\pm$ 0.00 <sup>c</sup>	2.11 $\pm$ 0.03 <sup>d</sup>	1.28 $\pm$ 0.02 <sup>c,d</sup>
142	nd <sup>a</sup>	> 22.00 <sup>a</sup>	nd <sup>a</sup>
1001	nd <sup>a</sup>	> 22.00 <sup>a</sup>	nd <sup>a</sup>
1017	nd <sup>a</sup>	> 22.00 <sup>a</sup>	nd <sup>a</sup>
1061	nd <sup>a</sup>	> 22.00 <sup>a</sup>	nd <sup>a</sup>
1063	0.12 $\pm$ 0.01 <sup>b</sup>	2.56 $\pm$ 0.14 <sup>b</sup>	1.15 $\pm$ 0.05 <sup>b</sup>
1121	nd <sup>a</sup>	> 22.00 <sup>a</sup>	nd <sup>a</sup>
1511	nd <sup>a</sup>	> 22.00 <sup>a</sup>	nd <sup>a</sup>
1567	nd <sup>a</sup>	> 22.00 <sup>a</sup>	nd <sup>a</sup>

In columns, different letter indicate significantly differences between strains for each variable ( $\mu_{\max}$ , G, OD<sub>max</sub>)

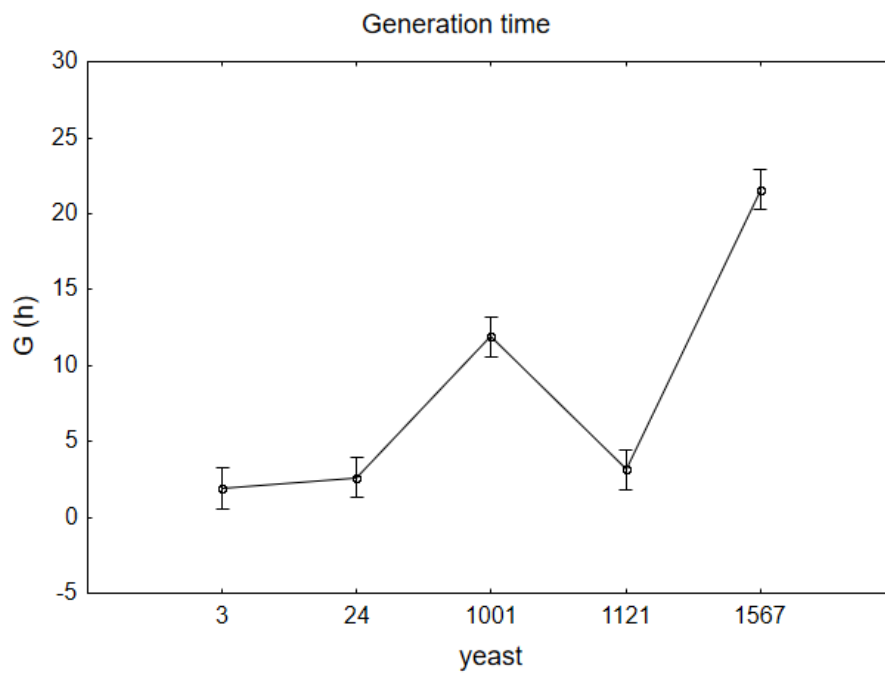


Figure 1

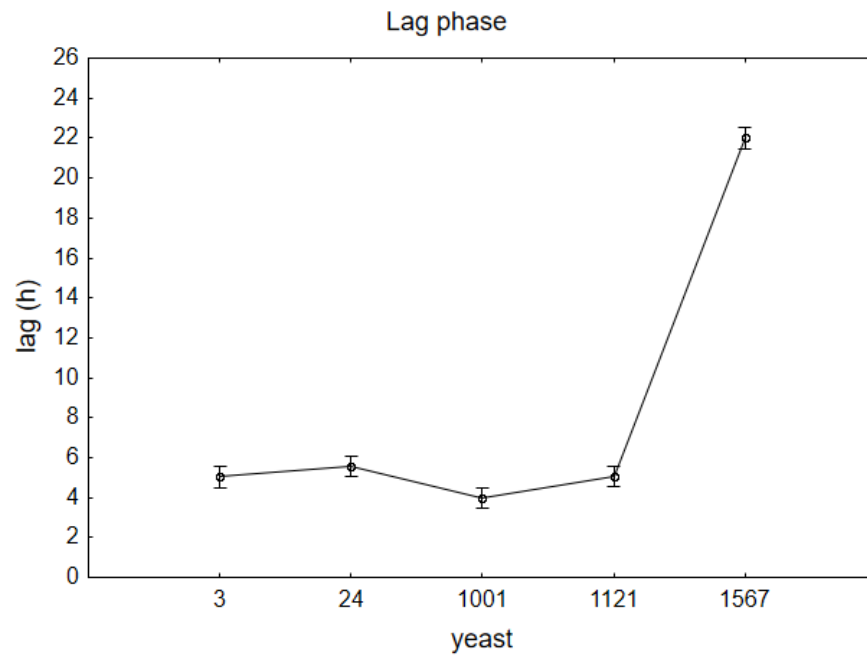
A



B



C



D

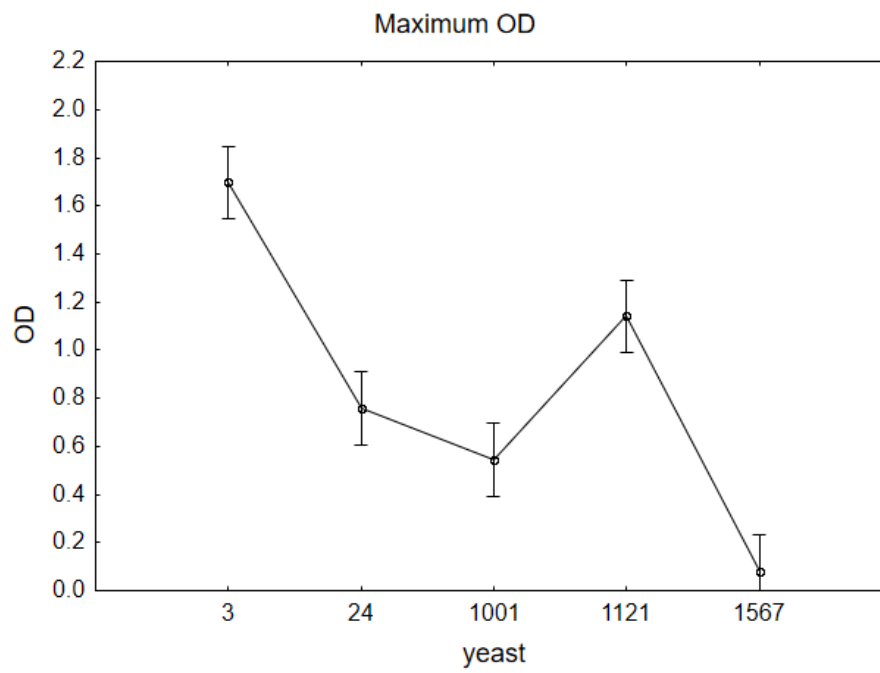
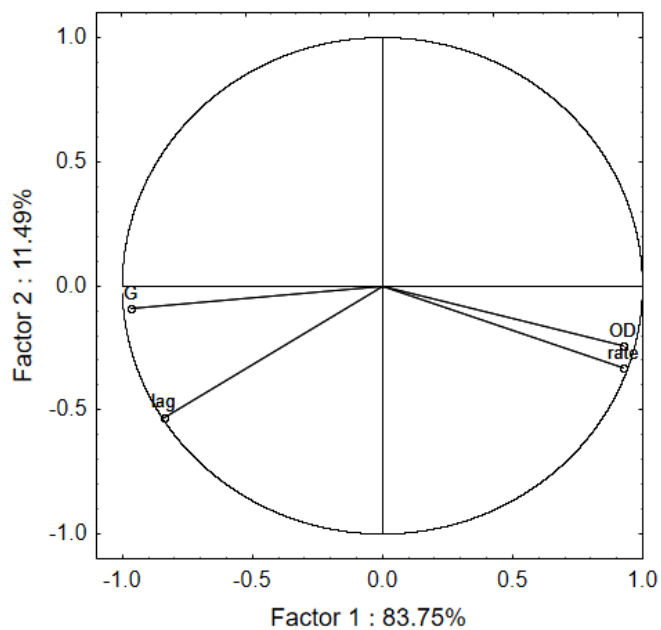


Figure 2

A



B

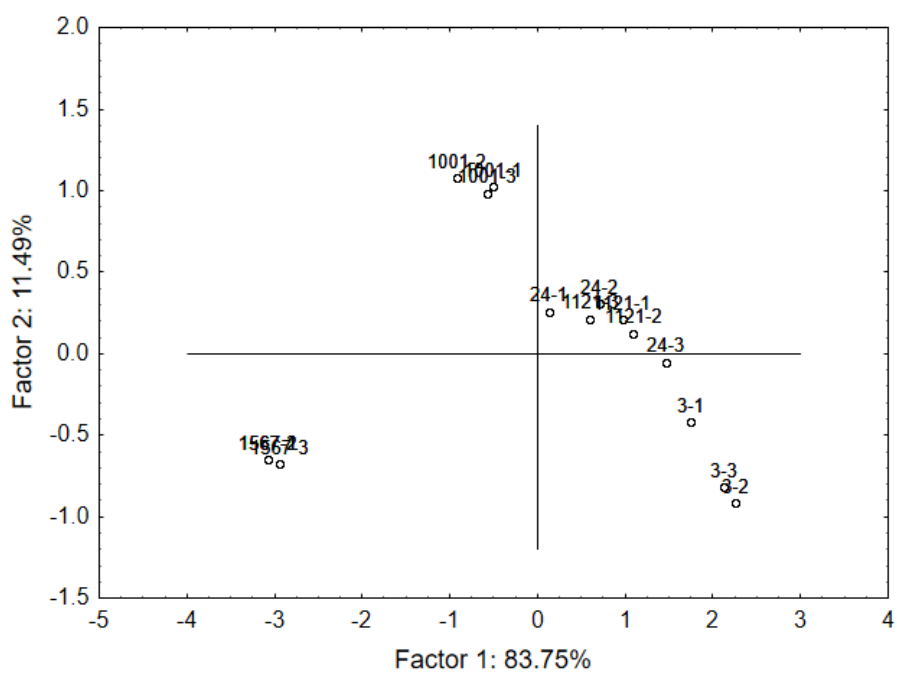
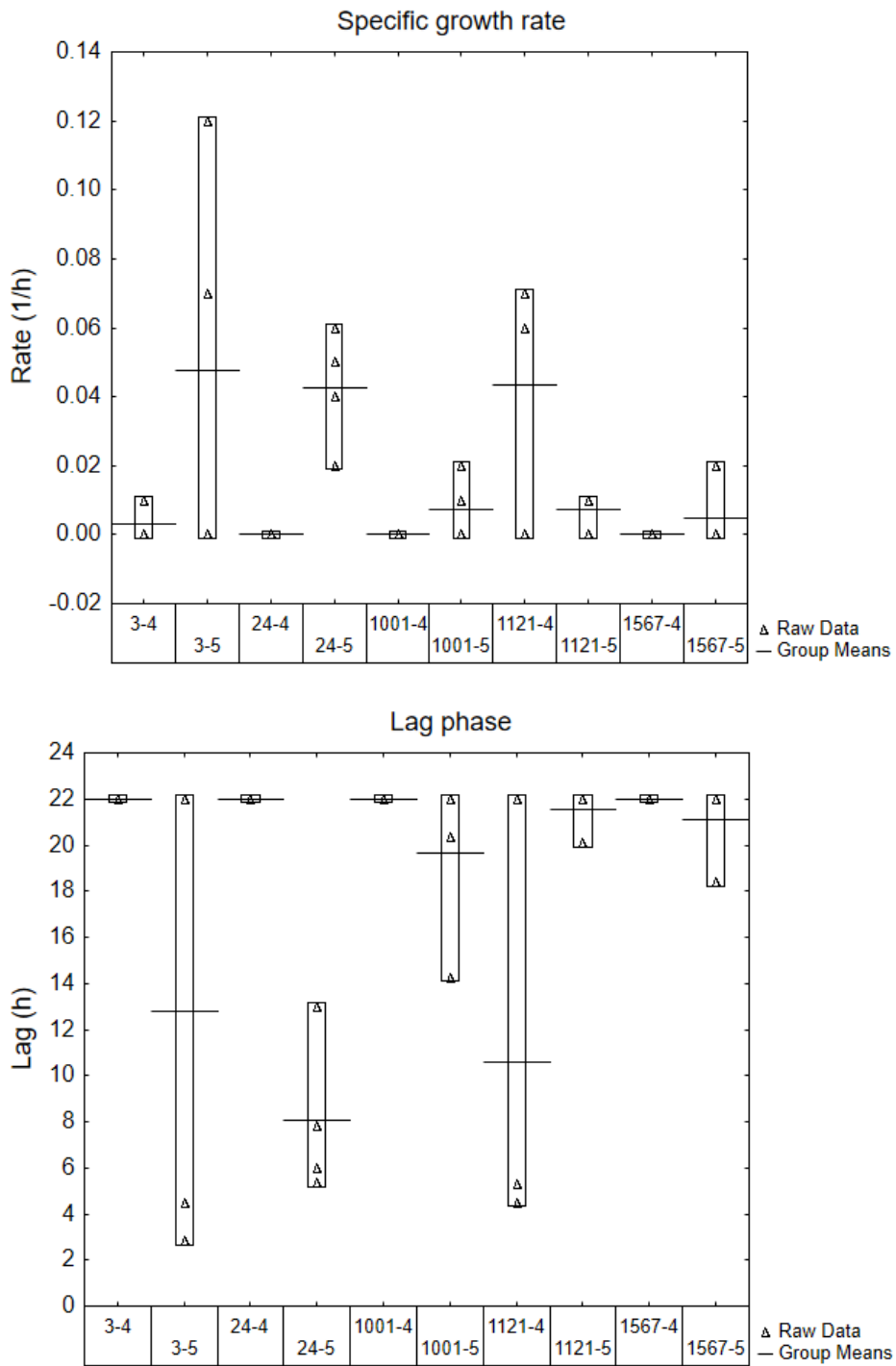


Figure 3



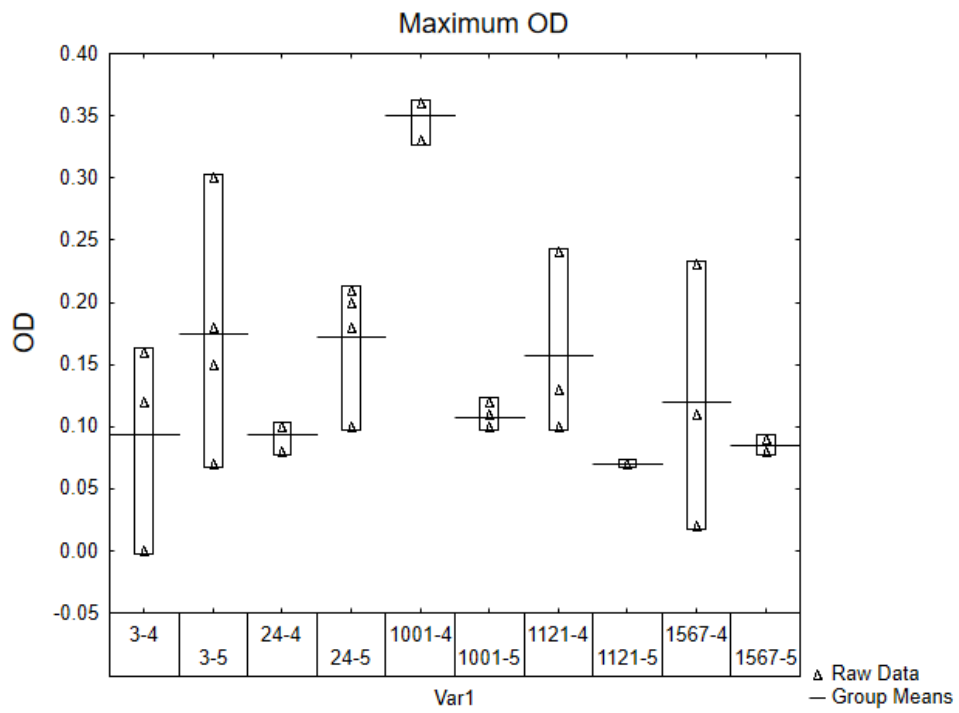
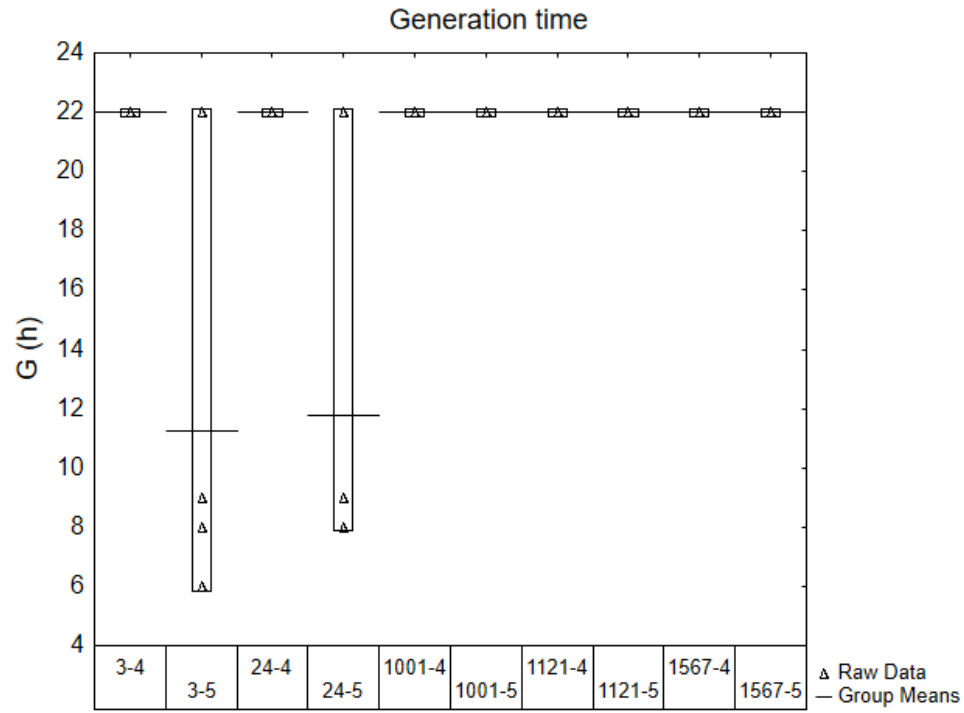
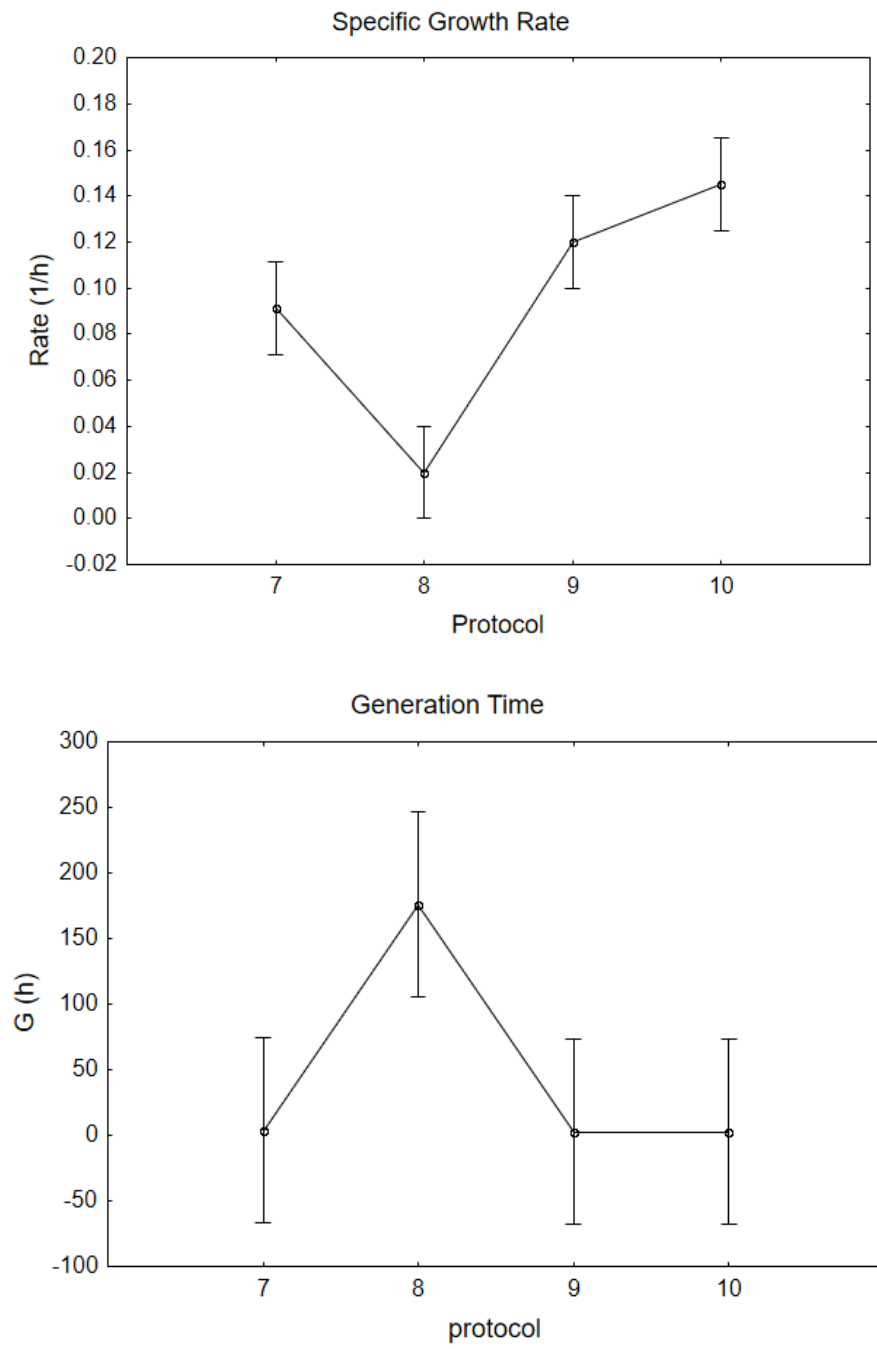


Figure 4



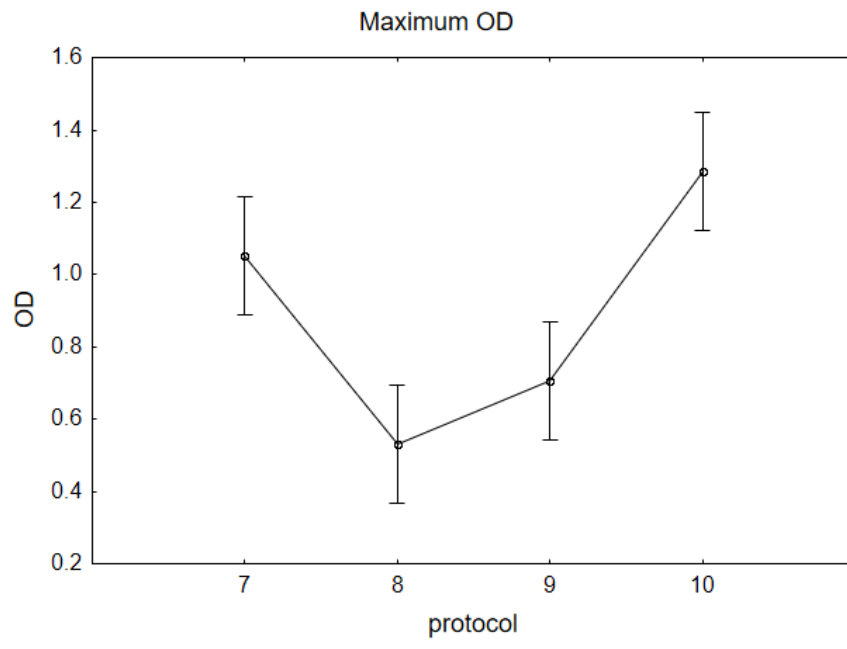


Figure 5

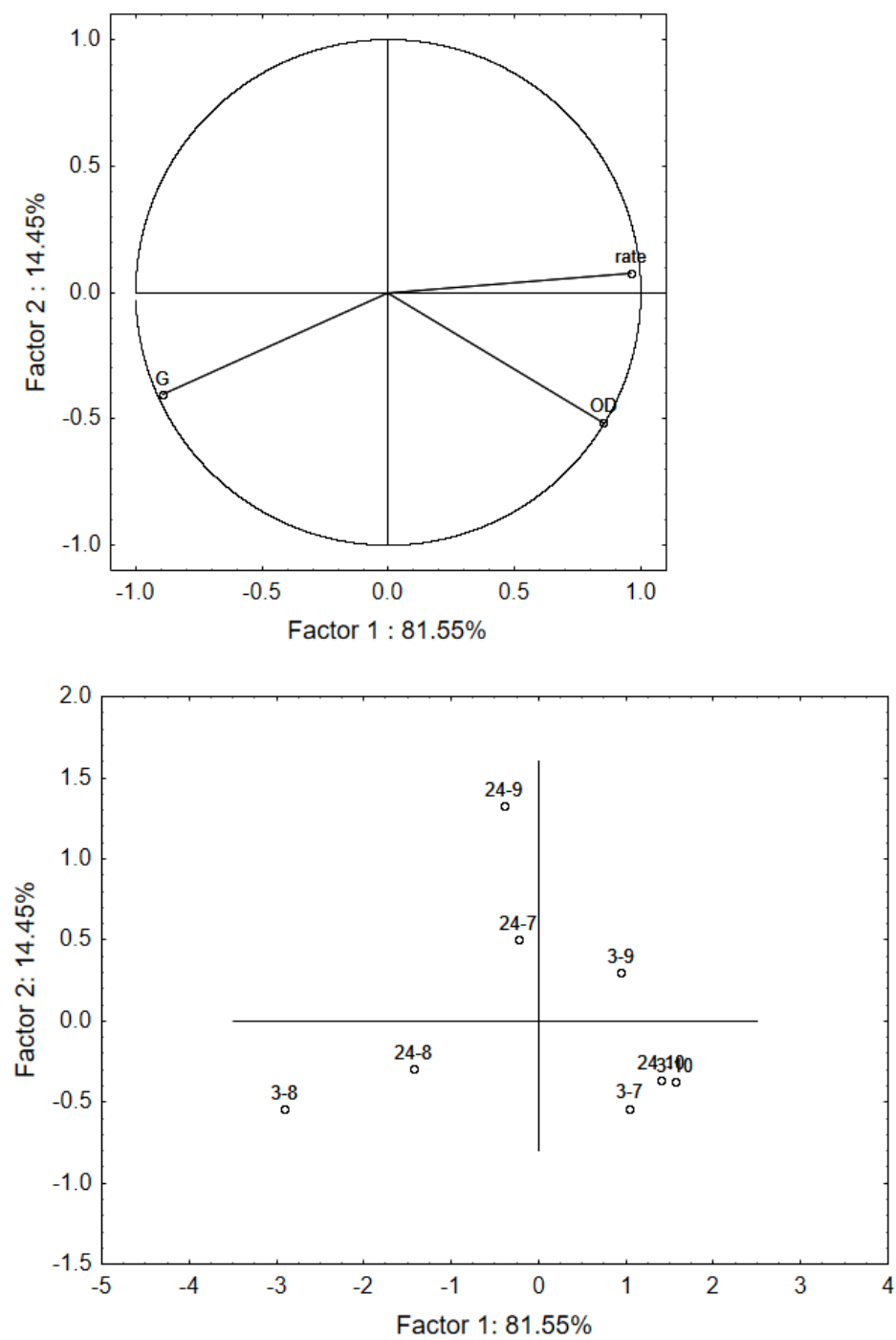
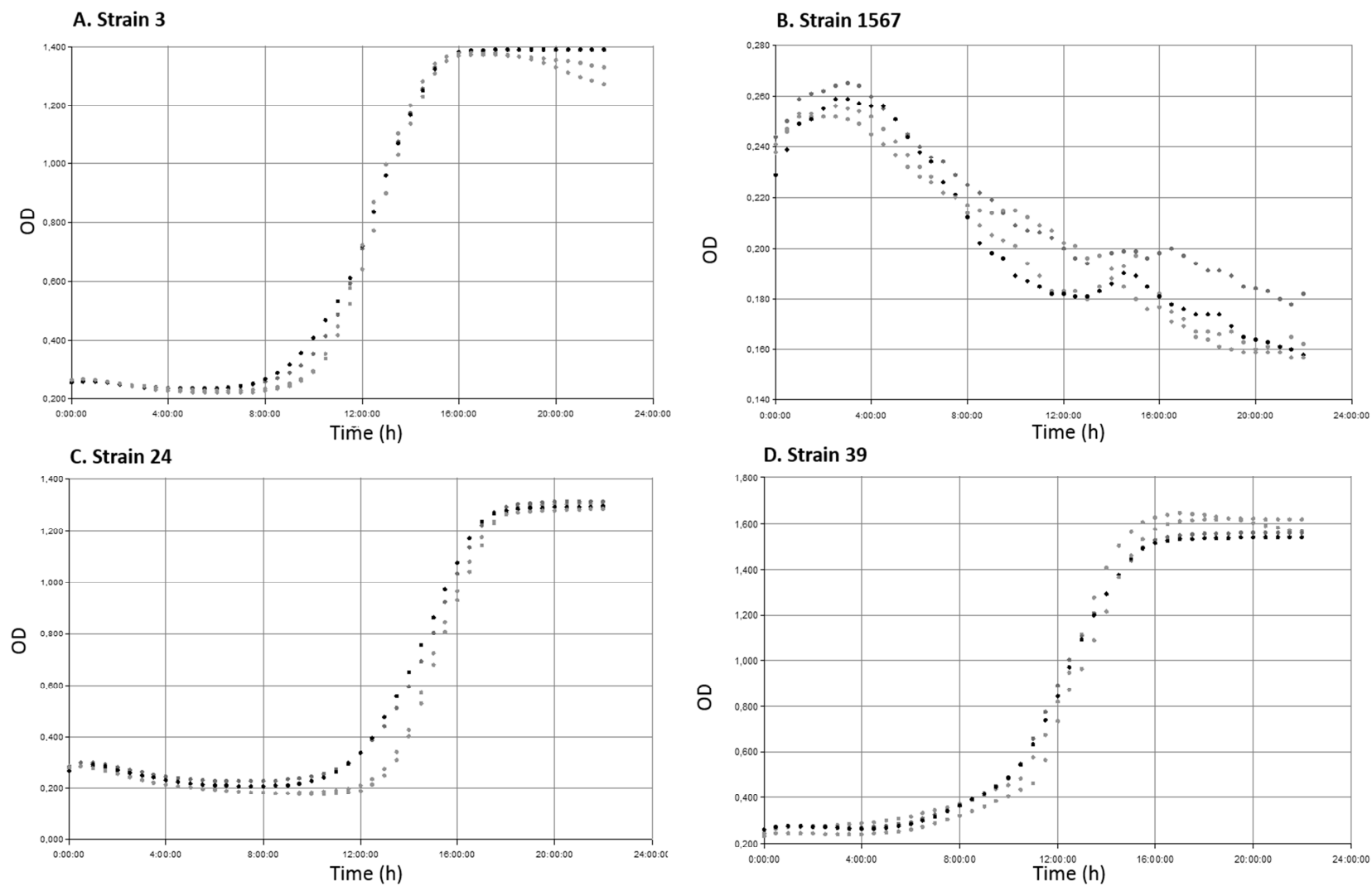




Figure 6



**1 Highlights**

2 New method for studying the survival/resistance of yeasts to the transit into the gut.

3 Statistical analysis of growth kinetic parameters of probiotic yeasts.

4 Marked probiotic character in *Saccharomyces* yeasts versus non-*Saccharomyces*.

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