Antimicrobial activity of yeast cell wall products against *Clostridium perfringens*

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Running Head: **YCWs effect on *Clostridium perfringens***

ABSTRACT

Yeast cell wall products (YCWs) are used worldwide as alternatives to antibiotics growth promoters for health and performances improvement in livestock. The success of yeast and YCWs as feed additives in farm animals' nutrition relies on their capacity to bind enteropathogenic bacteria**,** and on their immunomodulatory activity. *In vivo* studies report their anti-infectious activity on Gram-positive pathogens like clostridia. However, the *in vitro* antimicrobial activity of YCWs seems to be limited to some Gram-negative enteropathogens, and literature lacks*in vitro* evidences for antimicrobial effect of YCWs against *Clostridium perfringens*. This study aims to measure the antimicrobial activity of YCWs on *C. perfringens*. Five different YCWs were assayed for their capacity to inhibit the growth of *C. perfringens*, by analysing the growth kinetics of the pathogen. All YCWs inhibited the growth of the pathogen, by reducing the growth rate and the maximum growth value and extending the lag phase duration. The effect on the growth parameters was product- and dosage-dependent. The most effective YCW (namely YCW2), at the minimum effective concentration of 1.25mg/mL, increased the lag phase duration by 3.6h, reduced the maximum growth rate by more than 50%, and reduced the final cell count by 10^2 CFU/mL in 24h, with respect to the control. YCWs did not show a strain-dependent impact on *C. perfringens* growth when tested on different strains of the bacterium.

Keywords: yeast cell wall; antimicrobial; *Clostridium perfringens*; growth kinetics; feed; foodborne pathogens; food safety.

INTRODUCTION

Clostridium perfringens is a Gram-positive spore-forming obligate anaerobic bacillus widely distributed throughout the environment, and it is found in foods, sewage, soil, faeces, and gastrointestinal tract of healthy persons and animals (McClane, 2013). In poultry, *C. perfringens* is found in the intestinal content of healthy bird (Shimizu *et al*., 2002), in meat, on eggshells, paper pads and chicken dander in the hatchery (Craven *et al*., 2001). *C. perfringens* is one of the most important causes of foodborne disease in humans, and it is also commonly involved in several human and animal diseases, including gas gangrene, enterotoxaemia, food poisoning and necrotic enteritis (Collins *et al*., 1989; EU. 2003).

After EU ban in 2006 on the use of antibiotic drugs as growth promoters (AGPs) and for prophylactic purposes, antimicrobial additives are increasingly used in farm animals nutrition as substitutes of AGPs, and some natural substances are being used or researched for their antimicrobial effect, like prebiotics (inulin, fructo-oligosaccharides, mannan-oligosaccharides), probiotics (yeasts, lactic acid bacteria) and anti-infectious substances (Jouany*et al*., 2007; Murugesan *et al*., 2015; Ravindran, 2013; Stricklin*et al*., 2000; Zanini*et al*., 2015).

Yeast cell wall products (YCWs), derived from brewery or bakery yeast, are used worldwide as an alternative to AGPs concerning the promotion of health and performance in livestock (Fowler *et al*., 2015; Ghosh *et al*., 2012). The success of YCWs as growth promoters is based on their capacity to bind enteropathogenic bacteria (Ganner*et al*., 2013), and on their immunomodulatory activity (Kogan and Kocher, 2007). YCWs are suggested as anti-adhesive agents and are thus proposed to prevent attachment of pathogenic bacteria to the intestinal epithelium of the host, by interfering on the adhesion of bacterial fimbriae or afimbrial adhesins with surface lectins of the intestinal cells of the host, hence preventing the colonization of the intestinal mucosa by the pathogenic bacteria (Songer*et al*., 1996). Using the *in vitro* agglutination method and microscopy, different authors showed that mannan-oligosaccharides in YCW bind *E. coli, Salmonella,* and *Vibrio cholerae*

(Eshdat*et al*., 1978; Mirelman*et al*., 1980; Oyofo*et al*., 1989; Spring *et al*., 2000; Trevisi*et al*., 2012). A quantitative *in vitro* microplate assay was developed by Ganner*et al*. (Ganner*et al*., 2010; Ganner*et al*., 2013) and is based on the measurement of the optical density as growth indicator of adhering bacteria. The authors demonstrated that a purified YCW can differentially bind Gram-negative pathogens, such as some *E. coli* strains and *Salmonella* spp., but it did not bind Gram-positive bacteria of the genera lactobacilli and bifidobacteria, as well as *C. perfringens*. On the whole, all these *in vitro* results confirm the efficacy of YCWs as antimicrobial, thus reducing the amount of the pathogens that can adhere on the intestinal wall (Trevisi*et al*., 2012; Ganner*et al*., 2010; Pérez-Sotelo *et al*., 2005), and reveal that they may exert a selective effect against some bacteria while having no adverse effect on beneficial and commensal bacterial population. Recent *in vivo* reports highlighted the beneficial effects of yeast products in maintaining animal performances and health while minimizing mortality and morbidity when there is a *C. perfringens* challenge in an antibiotic-free production situation (Fowler *et al*., 2015; Hashim*et al*., 2018; M'Sadeq*et al*., 2015; Thanissery*et al*., 2010; Van Immerseel*et al*., 2004).

YCWs composition is very variable, depending on the species and strain they are originated, so it is not possible to make general considerations on their effect, but it is necessary to test *in vitro* their efficacy as antimicrobials against pathogenic bacteria (Ganner*et al*., 2013; Trevisi*et al*., 2012). So far, yeasts and derivatives are under-represented in the literature as anti-*C. perfringens* agents and more research is needed to confirm their effectiveness as antimicrobials versus strict anaerobic pathogens(Santovito*et al*., 2018). This study aims to evaluate the *in vitro* efficacy of selected yeast cell walls in inhibiting the growth of *C. perfringens* and paves the way to understand the mechanism underlying the antimicrobial activity of YCWs towards the pathogen.

MATERIALS AND METHODS

81 **Yeast cell wall products**

82 YCWs were provided by Phileo/Lesaffre International. Four out of five products contained cell wall
83 fractions derived from *Saccharomyces cerevisiae* (YCW1-4), while one was a mannoproteins
84 concentrate (MAN). The supplier declared that products were obtained using different production
85 methods. YCWs were analysed for microbial contamination by aerobes and anaerobes, according to
86 ISO methods (ISO7937. 2004; ISO4832. 2006; ISO4833-1. 2013).

87 **Bacterial strains and culture conditions**

88 *Clostridium perfringens* type C strains and isolates are listed in Table 1. All strains were grown at
89 37°C under anaerobic conditions in thioglycollate broth (TG, Biolife, Italy)in an 815PGB series
90 La Petite glove box (PlasLabs Inch), supplied with anaerobic 10% CO_2/10%Hydrogen/Nitrogen gas
91 mixture. In the TG broth we did not observe precipitation of YCWs and YCW:bacteria complexes.
92 Therefore,we used TG broth since it ensuredoptimal spatial interaction between cells and
93 antimicrobial compounds.
94 The inoculum density was standardized by photometric measurement at 600 nm wavelength using
95 anUltrospec 3100 Pro Spectrophotometer (Amersham, UK). The enumeration of *C. perfringens* was
96 performed by serial 10-fold dilutions in physiological saline and pour-plating on
97 TryptoseSulfiteCycloserine (TSC) agar (Biolife, Italy) according to ISO methods (ISO7937. 2004).
98 Strains were maintained in TG broth supplied with 20% glycerol and stored at −80 °C. Each strain
99 was analysed individually in all the experiments.

100 **Kinetic growth assays**

101 The experiment was designed to assess the inhibitory effect of YCWs on the growth kinetics of *C.*
102 *perfringens* ATCC 13124^T. Fowler *et al.* (2015) found that the optimal dose of YCW products
103 tested in their study was approximately 0.25-0.30 mg/mL. In order to verify if the same YCWs
104 concentration can have an antimicrobial effect *in* vitro, test tubes containing 10 mL of sterile TG
105 broth were supplemented with 0 to 10mg/mL of each YCWs. Three independent replicates were

prepared per each YCWs dosage and sampling time. Sampling times were 0, 6.5, 7.5, 9, 10, 13, 17, 20, and 24h. A mid-exponential phase culture of *C. perfringens* ATCC 13124^T at OD_{600nm}= 0.61 ± 0.14 was used for the inoculum, and duly diluted to have a final concentration of ca. 10^4 CFU/mL per each test tube. Blanks were prepared for each YCW dosage by adding sterile broth in place of the cell culture. Growth controls were prepared by inoculating the test strain in the absence of YCWs.

Samples were incubated in anaerobic conditions at 37 ± 1 °C up to 24 hours. At each time point, tubes were vortexed for 10 seconds and growth was monitored by measuring the absorbance at 600 nm. The net absorbance readings of each inoculated sample were obtained by subtracting the absorbance of the blank controls at each YCWs dosage from the absorbance of the respective test samples. At each time point, the corresponding Log[CFU/mL] count was measured by plate counting on TSC agar. The experiments were performed three times on three different days (n=9).

The antimicrobial effect of YCWs was further proven on other strains (Table 1), by measuring the CFU/mL reduction after 24 h. The results were expressed as the logarithm of the CFU count reduction per mg of YCW.

Data calculation and curve fitting

The OD_{600nm} values and the Log[CFU/mL] were plotted versus time (h). The Log[CFU/mL] data were analysed by non-linear regression analysis using the Baranyi and Roberts model (Baranyi and Roberts, 1994). The maximum specific growth rate μ_{max}(i.e. the specific growth rate at the exponential phase, h^{-1}), the maximum cell number y_{max} (Log[CFU/mL]), and the duration of the lag phase λ (h) were calculated using the Excel® macro DMFit, version 3.5 (www.ifr.ac.uk/safety/DMfit) (Baranyi and Roberts, Institute of Food Research, Norwich).

Inhibition rate

The inhibition rate (IR) (Li *et al*., 2016) was calculated using the following equation:

130 $IR = (\mu_0 - \mu_C)/\mu_0 = 1 - \mu_C/\mu_0$

131 Where μ_C is the specific growth rate at the concentration C of an YCW, and μ_0 is that of the control
132 in the absence of YCWs. IR_{50} is the concentration of YCW that causes 50% inhibition (i.e., IR =
133 0.5), and was derived from the inhibitory curves of IR as a function of YCW concentrations.

134 **Lag time extension**

135 To determine the effect of YCWs on the lag phase duration, we used a parameter termed "lag time
136 extension" (LE) that is defined as follows (Li *et al*., 2016):

137 $LE = \lambda_C - \lambda_0$

138 Where λ_C is the duration of the lag phase λ (h) of a microorganism at the concentration C (obtained
139 by DMFit analysis) of an YCW, and λ_0 is that of a control without the YCWs. In the scenario where
140 bacterial growth is completely inhibited, λ is regarded as infinite.

141 **Growth reduction**

142 The parameter *y*R was used to estimate the reduction in the y_{max} as a function of YCW
143 concentration. The *y*R parameter was calculated as follows:

144 $yR = 1 - (y_C - y_i)/(y_0 - y_i)$

145 Where y_C is the y_{max} obtained in 24h incubation, y_i is the concentration of the initial inoculum and y_0
146 is that of a control without the YCW. yR_{50} is the concentration of YCW that causes 50% reduction
147 (i.e., *y*R = 0.5) and was derived from the curves of *y*R as a function of YCW concentrations.

148 **Statistical analysis**

149 The growth parameters were analysed with All Pairwise Multiple Comparison Procedures (Tukey
150 post hoc test), with a 0.05 significance level. Growth parameters were correlated to YCWs

concentration using the Pearson correlation test at $p < 0.05$, and the SigmaPlot 12 software (Systat Software, San Jose, CA).

RESULTS

The chemical composition of YCWs as reported by the supplier is given in Table 2. YCWs did not differ substantially in chemical composition. The total viable aerobe count was negligible, being less than 3 CFU per mg of product (Table 2). Neither anaerobic bacteria nor *C. perfringens* isolates were found using the protocol indicated in ISO7973:2004. Blank controls that contained YCWs in TG broth did not show any growth in 24 h of incubation at 37°C in anaerobiosis. Therefore, it can be stated that endogenous microbial contamination did not interfere with the growth of the tested strains.

TG broth prevented the precipitation of bacterial cells and YCWs, in the overall incubation period of 24 h at 37°C. The TG broth was therefore selected as optimal medium, since it ensured a fast growth of *C. perfringens* and maintained cells and YCWs suspended, thus facilitating their interaction during the experiment.

On the assumption that a linear trend exists in the exponential growth phase (Hall *et al*., 2014), the absorbance values of the *C. perfringens* broth cultures (determined in the absence of YCWs, and in the exponential phase) were correlated to the respective CFU counts obtained by plate counting. A linear relationship was found between these values (R^2>0.99), and an optical density of 1.0 ± 0.05 corresponded to $9.30 \pm 0.21 \times 10^7$ CFU/mL . Growth curves obtained by plotting the growth data as OD_{600nm} and as Log[CFU/mL] count are shown in Figure 1. All Log[CFU/mL] data well fitted to the model of Baranyi and Roberts, with R^2 values > 0.97.

Examining the growth dynamics of *C. perfringens* over time, product- and concentration-dependent effects became evident. Even though all YCWs affected the growth of *C. perfringens*, the typical sigmoid curve shape was maintained. The curve fitting by the Baranyi and Roberts model allowed the calculation of the growth parameters y_{max}, λ, and μ_{max}. The analysis of *C. perfringens* growth kinetics in the presence of different YCWs showed that growth parameters were significantly affected ($p < 0.05$) by the products.

All YCWs reduced the μ_{max}during the exponential phase with increasing dosages of YCWs ($p < 0.05$) (Figure 2A). As a consequence of μ_{max} reduction, the inhibition rate (IR) increased with YCWs concentration in a different manner depending on the product (Figure 3A). Absence of growth was recorded using 10 mg/mL of YCW3 and YCW4, with IR values at 0.98 ± 0.01 and 0.95 ± 0.01. Pearson correlation test resulted in a negative correlation between the μ_{max} obtained in the presence of YCW3 and YCW4, and the relative concentrations ($r > -0.94$, $p < 0.001$) (Figure 2Aiii, 2Aiv). No correlation was found between μmax and YCW concentration for YCW1, YCW2, and MAN ($p > 0.05$) at YCWs concentrations higher than 1 mg/mL. The lowest concentrations of YCWs reducing by ≥ 50%*C. perfringens* growth (IC50) were 0.72 mg/mL for MAN, 1.18 mg/mL for YCW1, 1.31 mg/mL for YCW2, 4.76 mg/mL for YCW3 and 4.32 mg/mL for YCW4. Indeed, less than 1 mg/mL of MAN is necessary to slow down the growth in the exponential phase by 50%, whereas an amount ca. 6 times higher of YCW3 and YCW4 is necessary to obtain the same effect. These data show that YCW3 and YCW4 are less effective than MAN, YCW1, and YCW2 in reducing μ_{max}. The IR values calculated for YCW1 and YCW2 were comparable but higher than MAN.

At the concentrations used in this study, all YCWs significantly affected the λ value ($p < 0.05$) (Figure 2A). Extended lag phase (up to 13.6 h) was observed in *C. perfringens* cultures containing YCWs (Figure 1). The lag phase extension (LE) was in the range 0.0 – 3.7 h for YCW1 (Figure 2A-i); 3.6 – 5.9 h for YCW2 (Figure 2A-ii); 3.3 – 6.1 h for YCW3 (Figure 2A-iii); 3.1 – 6.5 h for YCW4 (Figure 2A-iv); and 0.8 – 4.4 h for MAN (Figure 2A-v). No correlation was found between LE and YCWs dosage ($p > 0.05$), except for MAN. A positive Pearson correlation coefficient ($p < 0.001$) and a linear relationship (linear regression with $R^2 = 0.962$) was found between LE and MAN concentration. To rank the YCWs we determined the minimum YCW concentration that gives at least 3 h increase in λ when compared to the control. These concentration values were 0.625 mg/mL for YCW1, YCW2, YCW3, and YCW4 (with LE values of 3.72 ± 0.21 h, 3.76 ± 0.64

h, 3.35 ± 0.15 h, and 3.12 ± 0.04 h respectively); and 5 mg/mL for MAN (LE = 3.17 ± 0.13 h). The LE/C value were calculated by dividing the LE values by the relevant YCW concentration. The parameter LE/C represents the delay in the lag phase that each product can induce per mg, expressed in h×mL/mg. These values were 5.95 ± 0.33, 6.02 ± 1.03, 5.36 ± 0.24, 4.99 ± 0.07 and 0.63 ± 0.03 h×mL/mg for YCW1, YCW2, YCW3, YCW4, and MAN, respectively. The most efficient products in extending the lag phase duration were YCW1 and YCW2, followed by YCW3, YCW4, with MAN resulting as the less effective.

As shown in Figure 2B, all YCWs affected the y_{max}, in a dose-dependent manner. A significant negative correlation between y_{max} and YCWs dosage was found ($R^2 > 0.936$, $p < 0.05$). Increasing dosages of YCW2 and MAN induced exponential decay of y_{max}values (Figure 2B). The curve obtained for YCW2 was sharper at the lowest dosages, proving the high efficacy of this product in inhibiting the growth of *C. perfringens*. Negative linear regression coefficients ($R^2 > 0.936$) were determined for YCW1, YCW3 and YCW4.

For the selection of the most efficient YCW in reducing y_{max}, we considered as effective the minimum YCW concentration reducing the y_{max} value by 50% in 24 h. As shown in Figure 3B, the yR_{50} values were 4.38, 2.35, 5.42, 4.55, and 7.81 mg/mL for YCW1, YCW2, YCW3, YCW4, and MAN respectively. Table 3 summarizes the results obtained by the analysis of the growth kinetic parameters.

Table 4 shows the values obtained for μ_{max}, λ, and y_{max} from growth curve fitting to the Baranyi and Roberts model, with the correlation between each value and the YCW dosage obtained by Pearson's correlation test and one-way ANOVA.

To confirm the YCWs inhibitory activity on *C. perfringens*, all products were tested towards four isolates with different origin, at the dosage determined as above. As shown in Figure 4, YCW2 confirmed its high efficacy in inhibiting the growth of *C. perfringens*. One mg of this product

228 reduced by *ca*. 2 Logs the final CFU count for Ad 1600, CP 56 and Ad 211b. The effect of YCW1
229 on bacterial growth was lower, being 1 Log CFU/mL count reduction per 1 mg/mL of product. The
230 YCW4, YCW3, and MAN were found as the less effective. Interestingly, YCWs did not show a
231 strain-dependent impact on growth inhibition.

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DISCUSSION

The success of YCWs as AGPs is due to the ability of their significant components - e.g., mannan-oligosaccharides and β-glucans - to prevent colonization by bacterial pathogens in the intestinal tract. YCWs have been proven to reduce the viability of specific bacterial pathogens and to increase the number of beneficial bacteria, with the consequent improvement of animal's performance and immunity (Kogan *et al*., 2007; Morales-Lopez and Brufan, 2013; Reisinger *et al*., 2012). The heterogeneous nature and diversity of YCWs makes it difficult to define *in vitro* assays that can be used to study the activity of these natural antimicrobial candidates. As a consequence, the central point to elucidate the efficacy and the mode of action of YCWs in preventing gut infections is the availability of accurate *in vitro* methods for studying the antimicrobial efficacy of YCWs on *C. perfringens*. The currently available test used for *in vitro* screening of YCWs is based on sedimentation and agglutination of the bacteria: yeast complexes, and by light and electron microscopy to visualize the binding of the bacteria (including *C. perfringens*) to YCWs (Ganan *et al*., 2012; Pérez-Sotelo *et al*., 2005; Posadas *et al*., 2017). Although providing the visualization of the interaction between YCWs and bacterial cells, this method did not allow the quantification of the extent of the inhibitory effect on the growth of target pathogens. In addition, the agar well/disc diffusion assays cannot be used with this kind of products, given the complex, tri-dimensional interaction of YCWs with the target bacteria in liquid medium.

Using the *in vitro* approach herein proposed, we studied the antimicrobial effect of YCWs on *C. perfringens* by analysing the effect of YCWs on the growth dynamics of the pathogen. The present study provides evidence on the inhibitory effect exerted by YCWs on *C. perfringens*, by direct counting of the residual Log[CFU/mL], and proposes a method that can be used to measure the direct effect of YCWs on the viability of the bacterium, granting the optimal growth conditions for the pathogen. In this study we demonstrated that the OD_{600nm} was directly correlated to the

Log[CFU/mL] count and that OD_{600nm} reduction in TG broth can be taken as an indicator for interaction and thus for antimicrobial effectiveness.

All YCWs assayed herein inhibited the growth of the pathogen by reducing the growth rate and the maximum growth value, and by extending the duration of the lag phase. The effect of YCWs on growth parameters was product- and dosage-dependent. LE was not affected by YCW concentration, unlike MAN. This may be due to the lower mannoproteins content in YCW1-4 than in MAN (mannoproteins concentrate), although no evidences of the direct implication of mannoproteins on the LE were found. YCWs did not show a strain-dependent impact on *C.perfringens* growth. One product out of five YCWs, namely YCW2, was selected as the most promising candidate for *C. perfringens* growth inhibition. Results on the effect of YCWs composition on the antimicrobial activity are not significant in this study, therefore further analysis are needed to identify components mainly contribute to the antimicrobial activity of YCWs.

To the best of our knowledge, this is the first *in vitro* study on the effect of YCWs on the growth kinetics of *C. perfringens*. Previous studies *in vivo* by Fowler *et al.* (2015) reported that the optimum dose for some YCWs was approximately 0.25-0.30 mg/mL, when these products were supplemented in starter broilers under an immune stress and *C. perfringens* challenge. Although the scenario is different, since *in vitro* studies do not reflect the conditions *in vivo*, it can be stated that the method proposed in this study can be useful to select the YCWs candidates to be used for *in vivo* studies.

Further studies are needed to elucidate the mechanisms underlying the growth inhibition effect exerted by YCWs on *C. perfringens* as well as on other anaerobic pathogens, which require specific set up of the growing conditions.

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388 ***Figure legends***

389 **Figure 1. Growth curves of *C. perfringens* in the presence of different concentrations of each**
390 **YCW.**

391 Growth data are reported as OD_{600nm} and Log[CFU/mL] and as a function of time (h) in the presence
392 and in the absence of YCW1-4 and MAN. Values are mean ± standard deviation (n = 9).

393 **Figure 2. Growth rate (μ), lag phase (λ), and y_{max} variation.**

394 The values obtained by the analysis of the growth rate (μ), lag phase (λ), and y_{max} variation are
395 reported as a function of YCWs concentration. Experimental points are mean ± standard deviation (n
396 = 9). A (i)-(v) are results obtained with different concentrations of YCW1-4 and MAN. B represent
397 y_{max} variation in the presence of different concentrations of YCW1-4 and MAN.

398 **Figure 3. Inhibition rate (IR) and reduction of y_{max} (yR) variation.**

399 The values obtained by the analysis of (A) the inhibition rate (IR) and (B) the reduction of y_{max} (yR)
400 are reported as a function of YCWs concentration. The grey line represents the ordinate value of the
401 constant IR=0.5 (A) and yR=0.5 (B) for the calculation of IR_{50} and yR_{50} values respectively. Data
402 points are mean ± standard deviation in three independent experiments (n = 9).
403

404 **Figure 4. Growth reduction of different *C. perfringens* strains induced by YCWs.**

405 Data represent the final cell density measured after 24 h of incubation by plate counting. The final
406 CFU/mL count was referred to the control and expressed as (Log) CFU count reduction per mg of
407 each YCW. The effect of YCWs on the growth of *C. perfringens* strains is represented as mean of
408 three replicates in three independent experiments. Error bars are standard deviations among
409 replicates.
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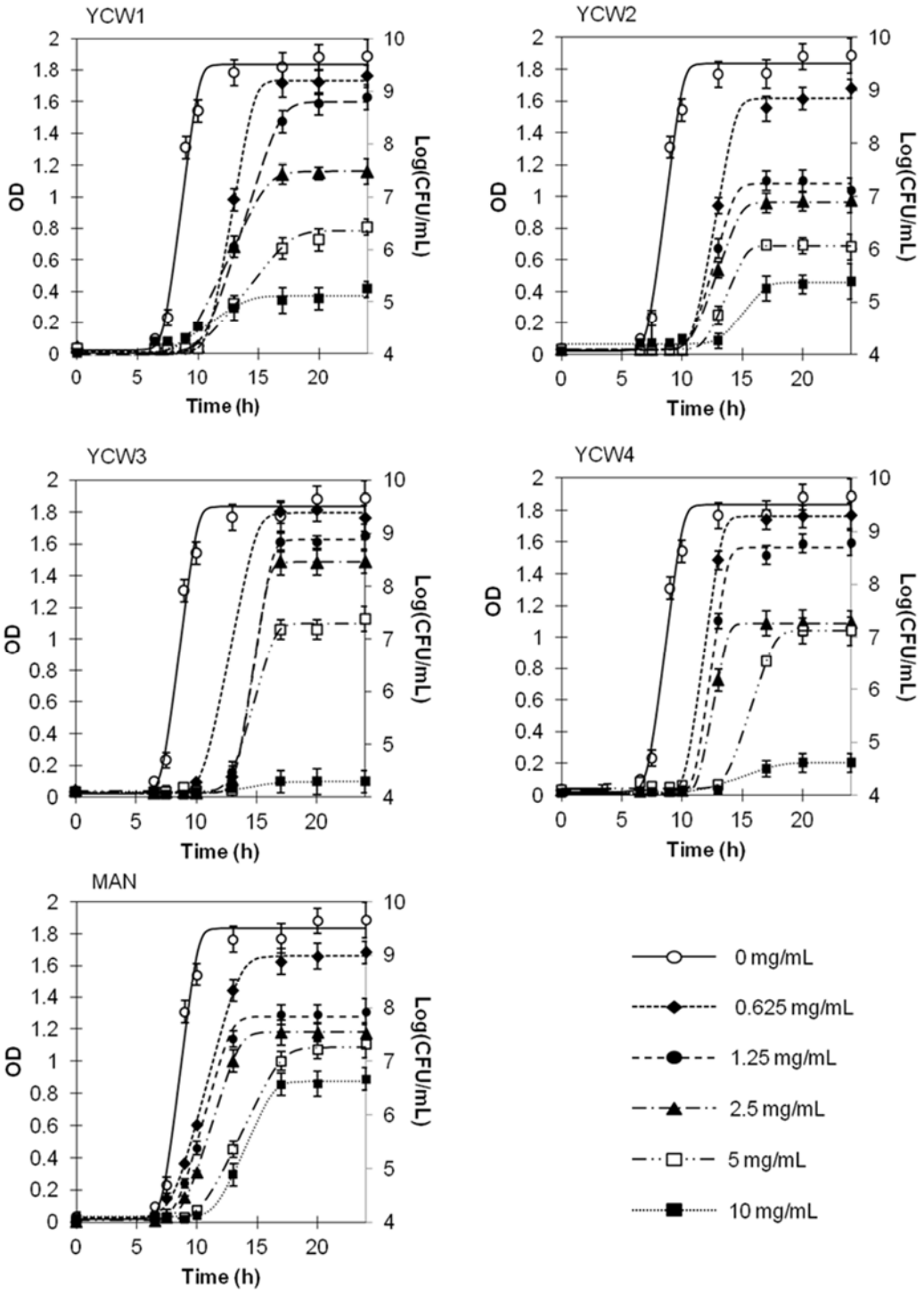

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413 Figure 1

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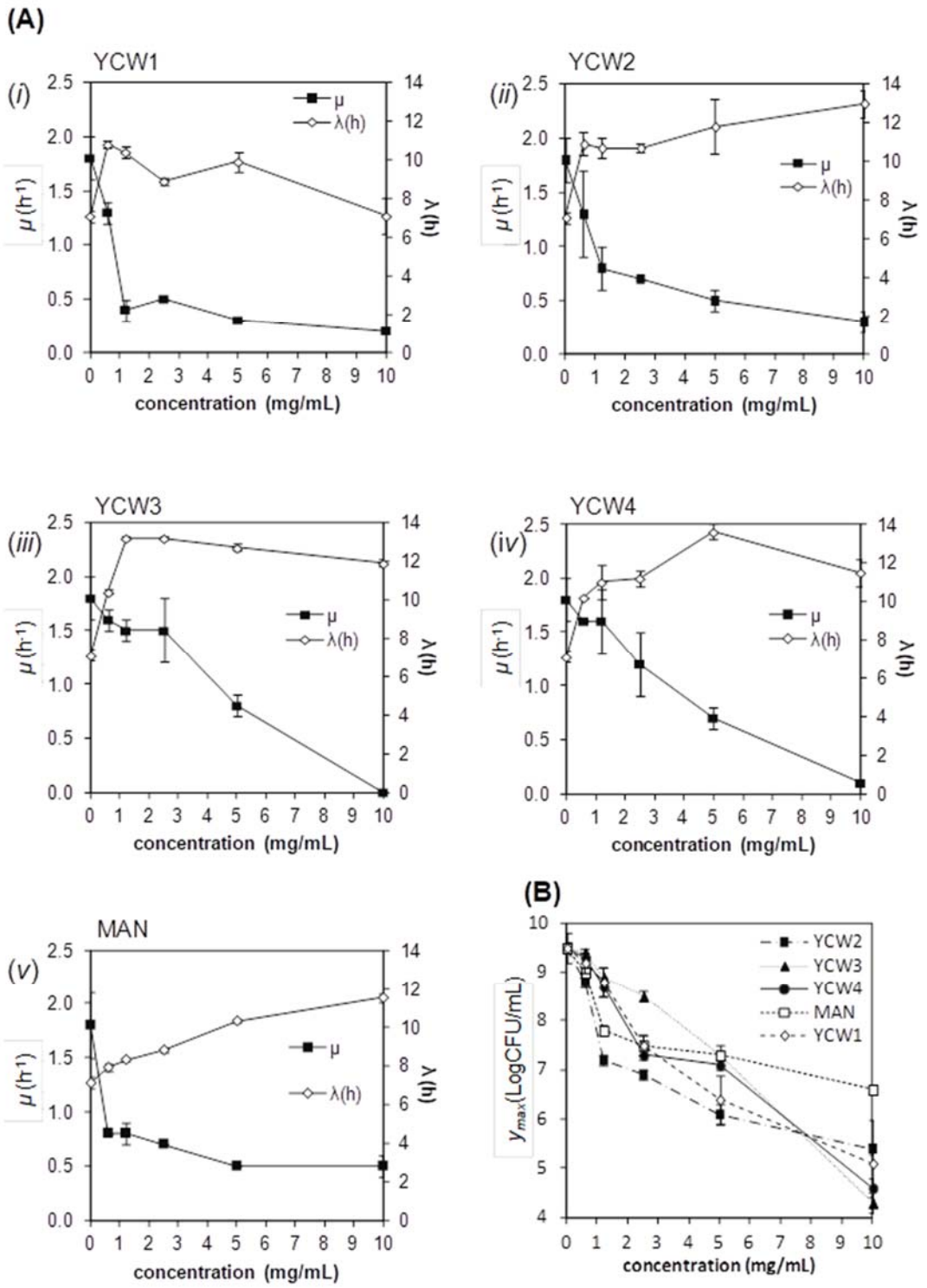

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416 Figure 2

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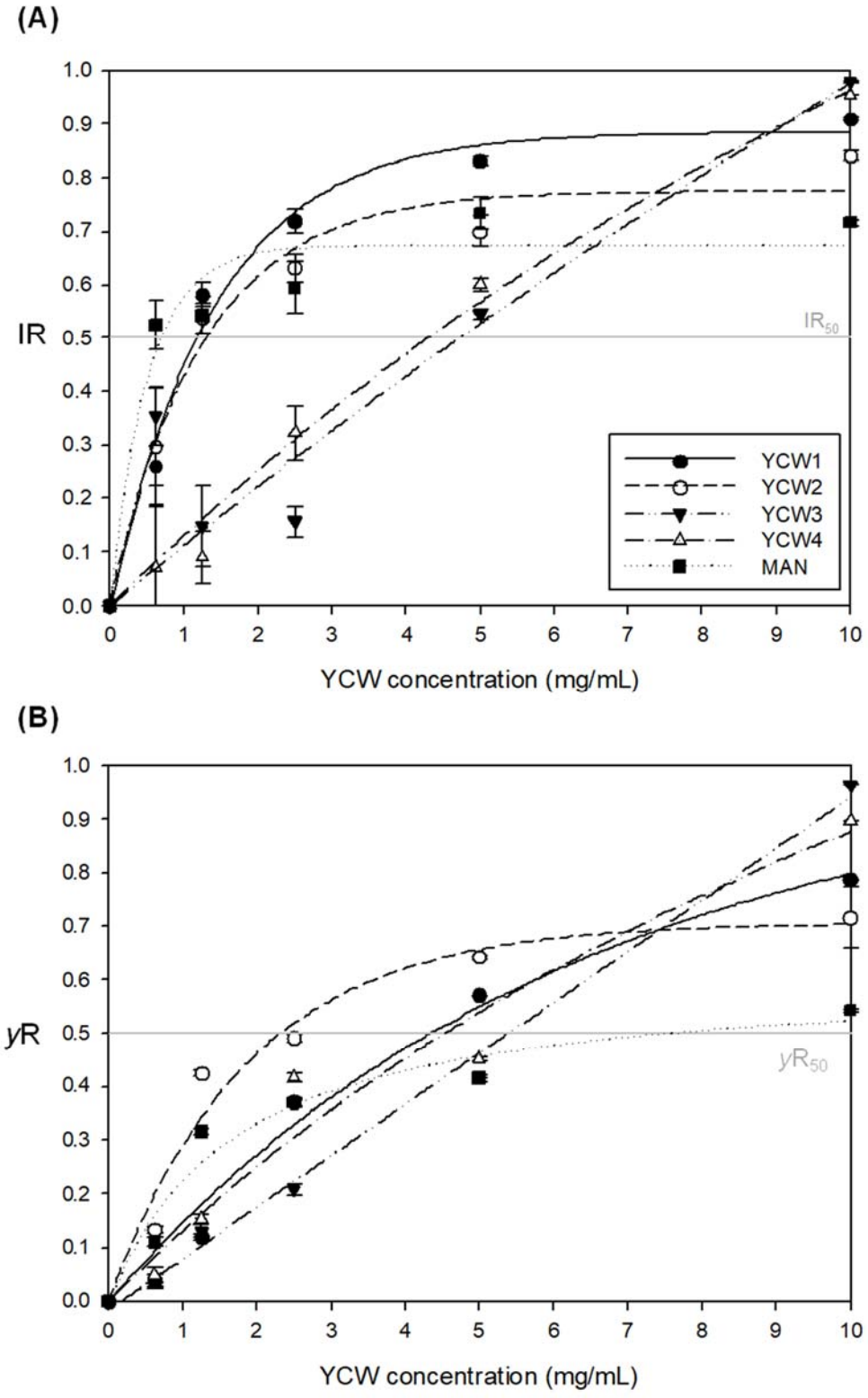

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419 Figure 3

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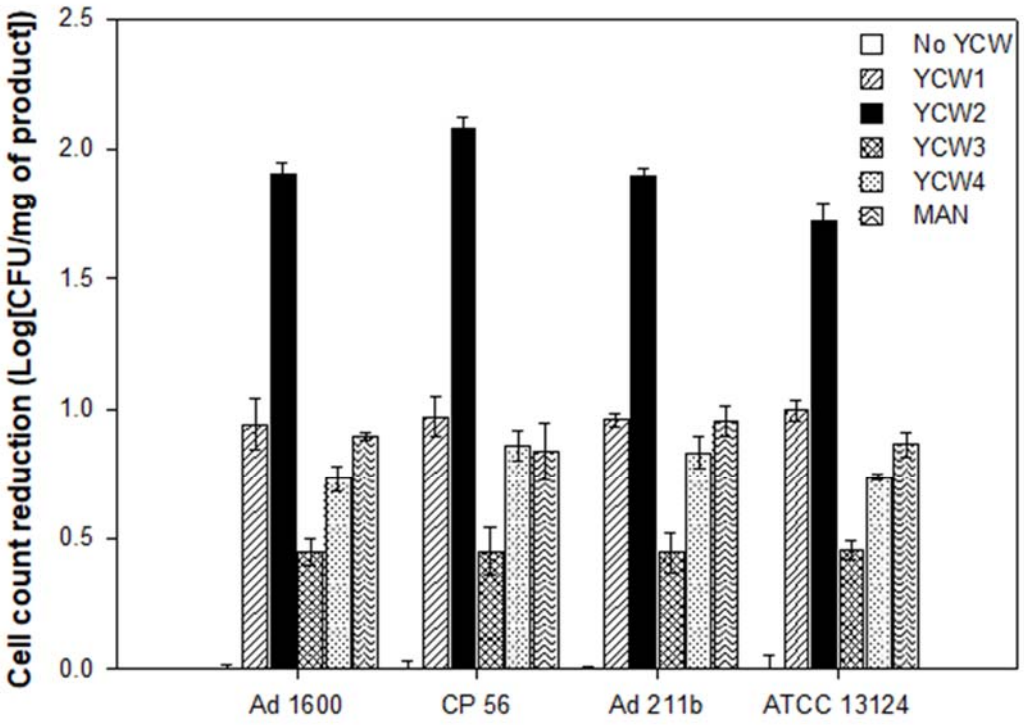

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422 Figure 4

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