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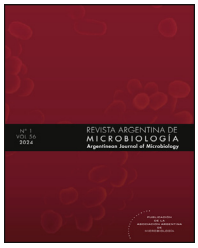

ORIGINAL ARTICLE

Inhibitory action of antibiotics on *Kluyveromyces marxianus*

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KEYWORDS
Kluyveromyces marxianus;
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Abstract A bioassay containing *Kluyveromyces marxianus* in microtiter plates was used to determine the inhibitory action of 28 antibiotics (aminoglycosides, beta-lactams, macrolides, quinolones, tetracyclines and sulfonamides) against this yeast in whey. For this purpose, the dose–response curve for each antibiotic was constructed using 16 replicates of 12 different concentrations of the antibiotic. The plates were incubated at 40 °C until the negative samples exhibited their indicator (5–7 h). Subsequently, the absorbances of the yeast cells in each plate were measured by the turbidimetric method (λ = 600 nm) and the logistic regression model was applied. The concentrations causing 10% (IC10) and 50% (IC50) of growth inhibition of the yeast were calculated. The results allowed to conclude that whey contaminated with cephalosporins, quinolones and tetracyclines at levels close to the Maximum Residue Limits inhibits the growth of *K. marxianus*. Therefore, previous inactivation treatments should be implemented in order to re-use this contaminated whey by fermentation with *K. marxianus*.

PALABRAS CLAVE
Kluyveromyces marxianus;
Test de inhibición;
Antibiótico;
Modelo logístico

Acción inhibitoria de los antibióticos sobre *Kluyveromyces marxianus*

Resumen Se utilizó un bioensayo en placas de microtitulación que contenían *Kluyveromyces marxianus* para determinar la acción inhibitoria en suero de 28 antibióticos (incluyendo aminoglucósidos, betalactámicos, macrólidos, quinolonas, tetraciclinas y sulfonamidas) contra esta levadura. Para ello, se construyeron curvas dosis/respuesta utilizando 16 réplicas de 12 concentraciones diferentes de cada antibiótico. Las placas se incubaron a 40 °C hasta el viraje del indicador de las muestras negativas (5-7 h). Posteriormente, se midieron las absorbancias

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de las células de levadura en cada placa por el método turbidimétrico (λ = 600 nm), y se aplicó el modelo de regresión logística. Se calcularon las concentraciones que causan el 10% (IC10) y el 50% (IC50) de inhibición del crecimiento de la levadura. Los resultados permitieron concluir que el suero contaminado con cefalosporinas, quinolonas y tetraciclinas en niveles cercanos a los límites máximos de residuos inhiben el crecimiento de *K. marxianus*. Por lo tanto, deberían implementarse tratamientos previos de inactivación para reutilizar sueros contaminados por fermentación con *K. marxianus*.

Introduction

Antibiotics are widely used to treat different diseases of dairy cattle[45]. Consequently, some antibiotic molecules can be eliminated in feces, urine and milk[17].

Whey, the by-product of the dairy industry obtained during cheese making, may contain residues of antibiotics such as penicillin[22], amoxicillin and ampicillin[19], erythromycin and oxytetracyclines[6,22,45], cefquinome and ceftiofur[19], ciprofloxacin[31] and tetracyclines[1].

Among the various industrial uses of whey, it is important to highlight the production of different products[24], such as biofuels[7,15], bioethanol[3,12,35,37,39–41], enzymes[5,32], higher alcohols with antibacterial–antifungal properties[2,10,11,33], beverages[18,20,26,38] and biomass[9,30,50,51], among others.

It should be noted that many of these industrial whey applications use yeasts, preferably *Kluyveromyces marxianus*, due to their ability to ferment lactose[4,14,21,28,34,35,40,48]. However, residues of antibiotics in whey at concentrations close to the Maximum Residue Limits (MRLs) established by the legislation[8,16] could inhibit the fermentative processes of *K. marxianus*.

Therefore, the objective of the present work was to analyze the inhibitory effect of antibiotics on the growth of *K. marxianus* through a simple bioassay in microtiter plates.

Materials and methods

A yeast inhibition bioassay in microtiter plates was developed according to the following stages:

1. *Yeast*: *K. marxianus* (ATCC 8554) was obtained from the strain collection of the Departamento de Microbiología of the Facultad de Ingeniería Química of the Universidad Nacional del Litoral, Santa Fe, Argentina.

Culture medium consisting of 5 g/l yeast extract (Merck Millipore, USA), 30 g/l casein peptone (Biokar Diagnostics, France) and 40 g/l lactose (Merck Millipore, USA) was adjusted to pH = 7.0 and sterilized at 121 °C for 15 min. Then, the medium was inoculated with *K. marxianus* and incubated at 40 °C for 24 h, in order to obtain cells in exponential phase (OD = 0.5, λ = 600 nm).

2. *Culture medium with yeast*: A semisynthetic matrix made up of antibiotic-free whey deproteinized by heat treatment at 120 °C for 20 min was used. Then, the culture medium was fortified under sterile conditions with 0.5% yeast extract (Merck Millipore, USA), 3% casein peptone (Biokar Diagnostics, France) and lactose (Merck Millipore, USA) in order to obtain a matrix containing 0.9% protein and 5.0% lactose. Furthermore, the pH was adjusted to 7.0 with NaOH 0.1 M. Subsequently, 20% suspension of *K. marxianus* (ATCC 8554) in exponential phase and 50 mg/l of bromothymol blue were added. It should be noted that, the acid–base indicator is used to visualize the endpoint of the incubation of *K. marxianus*.
3. *Antibiotic-fortified solutions in culture medium*: Aqueous solutions (1000 mg/l) were prepared for the following 27 antibiotics (Sigma Chemical Co., St. Louis, MO, USA): 3 aminoglycosides, 10 beta-lactams, 3 macrolides, 4 quinolones, 3 tetracyclines and 4 sulfonamides. For each antibiotic, 12 concentrations were prepared (Table 1S online Supplementary File) using the culture medium described in ''Materials and methods'' section.
4. *Yeast inhibition bioassay*: Two microtiter plates were used for each antibiotic and 16 replicates of the 12 solutions in whey according to ''Materials and methods'' section were tested. In each well of the microtiter plates, 200 μl of antibiotic-fortified medium solution was added using an electronic dispenser (Eppendorf Research® Pro, Hamburg, Germany). Subsequently, the plates were incubated at 40 °C until the negative controls turned from blue to yellow. Previous studies revealed that an adequate incubation time is between 5 and 7 h, since prolonging this time (*e.g.* 9 h) produces no modifications in the bioassay response.

Subsequently, the microtiter plates were measured at 600 nm using a Biotek EL800 reader (BioTek Instruments Inc., Winooski, Vermont, USA) and the Relative Absorbances (RA) in each bioassay were calculated.

The RA were analyzed using the Maximum Likelihood estimation method contained in the Logistic Regression

Table 1 Logistic equations that represent the effect of antibiotic concentrations on the growth inhibition of *Kluyveromyces marxianus*.

Antibiotics	Logit [*P*] = $\beta_0 + \beta_1$ [ATB]			IC50	MRL
	β_0	β_1	C%		
Beta-lactams					
Penicillin G	2.0971	−0.0064	86.2	330	4
Amoxicillin	2.4839	−0.0009	94.1	2680	4
Ampicillin	1.6418	−0.0006	82.3	2700	4
Cloxacillin	1.6090	−0.0014	86.7	1200	30
Oxacillin	2.3871	−0.0014	90.6	1700	30
Cefadroxil	2.4659	−0.0294	91.6	84	100
Cephalexin	2.8972	−0.0284	89.8	100	–
Cefoperazone	2.8371	−0.0156	93.0	180	50
Cefuroxime	1.9394	−0.0108	83.5	175	–
Ceftiofur®	3.6675	−0.0179	90.9	205	100
Aminoglycosides					
Streptomycin	3.5496	−0.0031	92.9	1140	–
Kanamycin	4.3517	−0.00036	95.4	12000	150
Neomycin	3.0491	−0.0025	89.4	1200	1500
Macrolides					
Erythromycin	2.1208	−0.00005	86.3	41000	–
Tilcomisin	1.9330	−0.00023	86.6	8200	50
Tylosin	3.3525	−0.0029	95.3	1160	50
Tetracyclines					
Chlortetracycline	2.3751	−0.0410	89.6	57	100
Oxytetracycline	3.1727	−0.0508	97.1	62	100
Tetracycline	3.2406	−0.0359	95.3	90	100
Quinolones					
Ciprofloxacin	6.9824	−0.0391	97.2	178	100
Enrofloxacin	2.1906	−0.0167	93.2	130	100
Marbofloxacin	1.7982	−0.0120	85.5	130	75
Norfloxacin	3.6517	−0.0177	96.1	200	100

Logit [*P*]: logistic model; β_0, β_1: parameters estimated by the model; ATB: antibiotic; C%: concordance percentage; IC50: concentrations producing 50% of inhibition of yeast growth (μg/l); MRL: Maximum Residue Limits (μg/l).

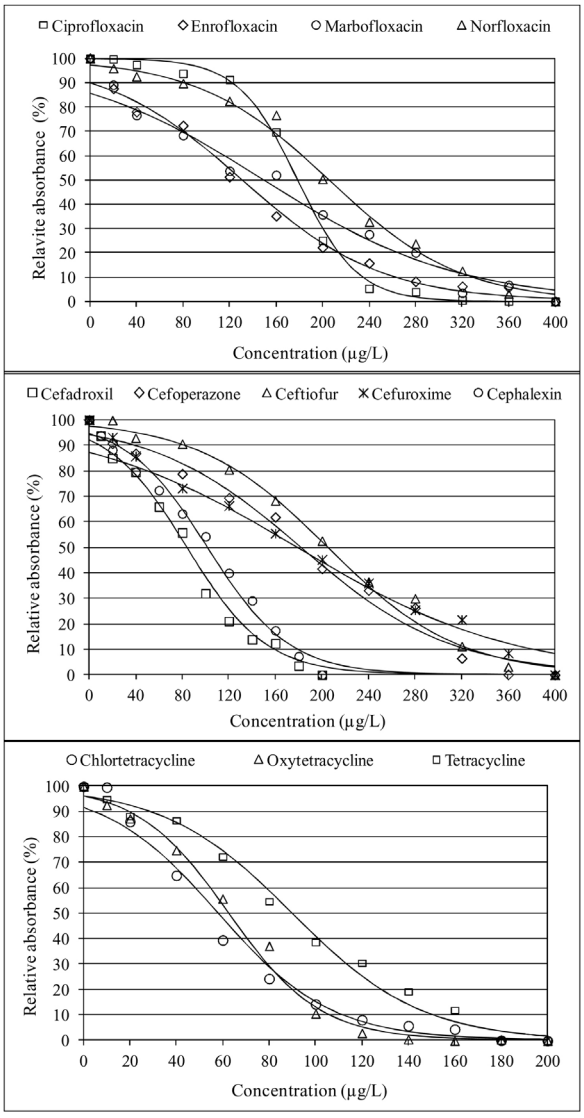

Figure 1 Effect of the cephalosporin, quinolone and tetracycline concentrations on the growth of *Kluyveromyces marxianus*.

procedure[46]. The following logistic regression model (logit) was used:

$$\text{Logit}_{ij}\left[\frac{\text{RA}}{(1\text{-RA})}\right] = \beta_0 + \beta_1[\text{ATB}]_i + \varepsilon_{ij} \quad (1)$$

where β_0 and β_1 are the parameters calculated with the logistic model; $[\text{ATB}]_i$ is the antibiotic concentration; and ε_{ij} is the model error.

For each antibiotic, IC10 and IC50 were calculated as the antibiotic concentrations that reduce 10% and 50% of RA[44].

Results

The application of the logistic regression model was adequate because the high concordance coefficients were between 82.3% for ampicillin and 97.3% for ciprofloxacin, as observed in Table 1. The values of coefficient β_1 represent the decrease in growth of *K. marxianus* due to the increase in antibiotic concentration. Thus, higher values of this parameter indicate greater inhibition by the antibiotic. The β_1 parameters for cephalosporins (from $\beta_{1,\text{ cefadroxil}} = -0.0294$ to $\beta_{1,\text{ cefuroxime}} = -0.0108$), quinolones (from $\beta_{1,\text{ ciprofloxacin}} = -0.0391$ to $\beta_{1,\text{ marbofloxacin}} = -0.0120$) and tetracyclines (from $\beta_{1,\text{ oxytetracyclin}} = -0.0508$ to $\beta_{1,\text{ tetracyclin}} = -0.0359$) were higher than those for the other antibiotics studied (Table 1). To visualize the growth inhibition of *K. marxianus* due to the concentrations of cephalosporins, tetracyclines and quinolones, dose–response curves were constructed (Fig. 1).

IC50 (μg/l) values in whey were close to those of MRLs (μg/l) in milk for cephalexin (100 *vs* 100), cefoperazone (180 *vs* 50), ceftiofur (205 *vs* 100), chlortetracycline (57 *vs* 100) oxytetracycline (62 *vs* 100), tetracycline (90 *vs* 100), ciprofloxacin (178 *vs* 100), enrofloxacin (130 *vs* 100), marbofloxacin (130 *vs* 75) and norfloxacin (130 *vs* 100). In contrast, IC50 values of penicillins, aminoglycosides,

macrolides and sulfonamides, except neomycin (Table 1), were very high compared to their MRLs (>30 MRLs).

Discussion

With respect to cephalosporins, Hamilton-Miller[23] observed growth inhibition of some pathogenic yeasts and filamentous fungi when analyzing semisynthetic cephalosporins that possess an N-benzyldithiocarbamate side group. Furthermore, an *in vitro* study to determine the inhibition of yeasts reveals the use of high concentrations (16 000 μg/ml) of a combination of cefoperazone sodium, sulbactam sodium, and cefradine in proportions of 2:2:3[29].

In the case of fungal species, Sanyal et al.[42] reported that the degradation products of cephalosporins inhibit the growth of *Trichophyton mentagrophytes* (dermatophytes) and *Macrophomina phaseolina* (a plant pathogen), but are not effective against *Candida albicans* (an opportunistic yeast) or *Aspergillus niger* (a saprophyte).

Therefore, the differences in sensitivity observed between cephalosporins and penicillins (Table 1) in this study could be attributed to the production of penicillinase by *K. marxianus*, which reduces the effectiveness of penicillins, thus enabling normal yeast fermentation.

With regard to quinolones, growth inhibition of *K. marxianus* by these antibiotics (Fig. 1) can be attributed to their effect on the topoisomerase enzyme that participates in the relaxation of the DNA double helix. Zhang et al.[52] described genotoxic effects against non-target species due to the binding of quinolones to topoisomerase. Similarly, Stergiopoulou et al.[47] reported growth inhibition of *Candida* spp. by fluoroquinolones.

Moreover, in an *in vivo* study involving fluoroquinolones, Dalhoff[13] emphasizes the effectiveness of topically administered moxifloxacin and gatifloxacin in treating infections caused by *Candida* spp. Additionally, in an *in vitro* study using a paper disk diffusion test, the author observes that gatifloxacin and sparfloxacin inhibit *Trichophyton rubrum*, *Fusarium solani* and *C. albicans*.

The *in vitro* study involving *Fusarium* spp. isolates, conducted by Kawakami et al.[27], reported inhibitory effects at high concentrations of fluoroquinolones: 750 mg/l of gatifloxacin, 312.5 mg/l of levofloxacin, and 1250 mg/l of moxifloxacin.

Finally, regarding tetracyclines, their effect on *K. marxianus* growth (Fig. 1) can be attributed to the inhibition of yeast protein synthesis, since these substances interfere with the binding of aa-tRNA to the 30S ribosomal subunit[49]. In this regard, Oriel and Waterworth[36] highlighted that minocycline can inhibit the growth of the yeast *C. albicans*, while Schwartz et al.[43]. suggested that the combined effect of polymyxin B and tetracycline can inhibit the growth of *C. albicans* and *Saccharomyces cerevisiae*. Similarly, Hooper et al.[25]. observed synergism in the inhibition of *Candida* spp. when using combinations of doxycycline with fluconazole and tigecycline with fluconazole.

Conclusion

In summary, a simple, fast (6 h) and low-cost bioassay was developed to analyze the inhibitory effect of antibiotics on the growth of *K. marxianus*. Results showed that whey contaminated with cephalosporin, quinolone or tetracycline residues at levels close to the MRLs in milk inhibits *K. marxianus* growth.

Therefore, whey contaminated with antibiotics at levels accepted by the legislation must be treated before fermentation with *K. marxianus*. Additionally, bioassays utilizing this methodology could be developed to determine the *in vitro* inhibition of pathogenic yeasts and fungi, aiming to reduce response times (5–7 h) compared to the current radial diffusion methods in Petri dishes. These future bioassays in microtiter plates could provide a rapid response (to antibiotics and/or antifungals, including their interactions) for patients with infections caused by fungi and/or yeast.

Conflicts of interest

The authors declare that they have no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ram.2023.12.004.

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