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Growth inhibition of bacterial isolates recovered from two types of Portuguese dry smoked sausages (*chouriço*)

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ABSTRACT

Potassium sorbate (PS), sodium benzoate (SB) and methyl *p*-hydroxybenzoate (MHB) were investigated as surface treatments for their ability to inhibit the growth of 18 isolates of spoilage and pathogenic bacteria from two types of Portuguese dry smoked sausages (*Chouriço*). MHB significantly inhibited the growth rate of 12 of the isolates ($p < 0.05$) whereas no effect was observed for four isolates of lactic acid bacteria, identified as *Enterococcus faecium*, *Pediococcus acidilactici* and *Lactobacillus curvatus*, and two isolates identified as *Clostridium aminovalericum* and *Staphylococcus epidermidis*. PS and SB had less influence on the bacterial growth rates. It was concluded that MHB can be applied as surface treatment to improve the stability and safety of the product along shelf life period in modified atmosphere package.

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1. Introduction

Potassium sorbate (PS), sodium benzoate (SB) and methyl *p*-hydroxybenzoate (MHB) are preservatives used as antimicrobial agents in the food industry (Chichester & Tanner, 1972; Eklund, 1989; Jay, 2000; Lueck, 1980; Pierson & Smoot, 1982; Sofos, 1989). They are allowed to be used in meat products (European Community legislation) '*quantum satis*' (i.e. without specified maximum level, these additives should be used no more than according to good manufacture practice) as surface treatment to dry sausage casings (Directive of the Council no. 96/85/CE; Directive of the Parliament 95/2/CE). Immersion of the casings in the preservatives may result in variable amounts of residual levels of the preservatives in the product. A few studies have analysed residual amounts of these preservatives in meat products under different conditions (McMeekin, Pennington, & Thomas, 1984; Myers, Edmondson, Anderson, & Marshall, 1983; Robach & Ivey, 1978; Stamm, 1985; Zamora & Zaritzky, 1987). The residual levels vary with the method of application, period of exposure, salt concentration, type of food, porosity of the food, shape and size of the food, and handling after exposure to the additive (Sofos, 1989). Matos, Barreto, and Bernardo (2005a) studied the residual amounts in dry smoked Portuguese sausages (*chouriços* type Alentejano and type Ribatejano) after immersing the natural casings for 15 min in 2.5% solutions of the preservatives. Residual amounts of the preservatives obtained in the final product varied between 0.01% and 0.06%. The diffusion of these preservatives from the surface into the product interior is inevitable. It is interesting to investigate the effect of these additives at the concentrations founded in the product and also in higher concentrations in order to evaluate which concentrations will be most effective on the spoilage and pathogenic bacteria of these meat products. Several studies have been carried out on the antimicrobial effects of the above mentioned additives (Draughton, Sung, Mount, & Davidson, 1982; Lueck, 1980; Sofos & Busta, 1981, 1983; Sofos, Pierson, Blocar, & Busta, 1986; Warth, 1985). However, the concentrations of these preservatives necessary for complete bacterial inactivation or for partial bacterial inactivation are different. Furthermore, it is necessary to consider interactive factors like bacterial species and strains, contamination levels, product composition, water activity, pH, the presence of other additives, physical treatment or processing, storage temperature, length of storage, storage atmosphere, and type of packaging (Pierson & Smoot, 1982; Sofos & Busta, 1981, 1983; Sofos et al., 1986). There is, however, a lack of information about the effects of these salts on growth of the spoilage bacteria that will remain during the storage period in a modified atmosphere package and that would decrease the shelf life and compromise product safety.

Since the application of these preservatives as surface treatments is currently not a normal practice in portuguese dry smoked sausages, effects of PS, SB and MHB were investigated for their

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ability to inhibit growth in different concentrations under optimal laboratory growth conditions of spoilage and pathogenic bacteria recovered from two types of Portuguese dry smoked sausages (*chouriço* type Alentejano and type Ribatejano) after the end of the customary shelf life period (120 days at 20 ± 5 °C) in modified atmosphere package (55% N_2, 45% CO_2). Studies were performed to investigate if these preservatives have indeed a clear inhibitory effect (which would be more effective and in which concentrations) on growth rate of bacterial isolates which have survived to packaging and storage conditions.

2. Materials and methods

2.1. Isolates, growth conditions and sampling

Bacterial isolates were recovered from Portuguese *chouriços* type Alentejano (A) and Ribatejano (R) at the end of the producer-defined shelf life period (120 days at 20 ± 5 °C in modified atmosphere package of 55% N_2 and 45% CO_2), produced by a large factory in Portugal. The sausages consisted of a mixture of pork meat and fat coarsely ground (to approx. ∅ 20 mm) with the ingredients listed in Table 1. After mixing, the batter was kept for one day at 5 °C. The batter was stuffed into salted pork intestine (Ribatejano type sausage) and into beef dry casing (Alentejano type sausage) and then dried and smoked for about three days (weight loss ca. 40%). The thermal and smoking treatment was divided in two phases. The first phase was conducted in a smoke chamber with temperature, relative humidity (RH) and smoke addition automatically controlled. This first phase included (1) 60 min at 50 °C, (2) 90 min at 53 °C and (3) 90 min at 55 °C. During these treatments smoke was given continuously. In the second phase, product was submitted to one traditional chimney at the same factory in which smoke was produced from firewood (oak wood). The product was stabilised for one day at 17–19 °C and at 75–85% (RH) before packaging. The packaging material was Combitherm film (70 mm-width and 300 mm-length), composed by polyamide/ethylene vinyl alcohol/polyethylene/Silica (PA/EVOH/PE/SY) – coextruded, laminate. Oxygen transmission rate (OTR; cm^3/m^2 day atm) of this film is (i) 0.01–0.02 for EVOH layer (at 25 °C and 0% of relative humidity (RH)); (ii) 460 and 600 cm^3/m^2 day - atm, at 25 °C and 0% of RH for PE layer; (iii) (Zagory, 1995) and, (iv) 30–110 cm^3/m^2 day atm, at 23 °C and 0% of RH for PA layer (Salgueiro, 2001).

Table 1
Composition of Alentejano and Ribatejano dry smoked sausages

Formulation (g/kg)	*Chouriço* Alentejano	*Chouriço* Ribatejano
Meat raw material		
Pork belly boneless, without rind	172.1	176.0
Pork picnic, boneless, without shank	688.2	528.1
Pork trimming 90/10	–	176.0
Sub-total	860.3	880.1
Ingredients		
Commercial sausage sodium polyphosphate, E452(i); (50% of P_2O_5)	5.0	5.0
Water	31.0	12.7
Sugar (dextrose)	2.1	2.1
Olive oil	5.2	–
Liquid smoke	1.0	1.4
Spice lourer	0.02	0.04
Sweet chilli	–	5.0
Powder clove	–	0.1
Garlic paste	11.0	11.3
Pimenton paste	4.3	8.8
Special pimenton paste	44.9	46.1
Hot pimenton paste	12.9	2.5
White pepper (powder)	1.7	1.1
Sodium chloride with sodium nitrite, E250, (0.6% of $NaNO_2$)	10.3	10.0
Salt	10.3	10.0
White wine	–	3.9
Sub-total	139.7	119.9
Total	1000	1000

The study was based on 12 samples (each sample was composed of a mixture of three sausages, with a total of 36 sausages) randomly drawn from two batches. Sausages were packed separately in modified atmosphere with a net weight of about 225 g and stored at 20 ± 5 °C for 120 days for further analysis. Sausage diameters varied between 40 and 50 mm.

The pH mean values after preparation of *Chouriço* sausages were 5.80 ± 0.17 and of 5.70 ± 0.10 and water activity between 0.93 ± 0.01 and 0.94 ± 0.01 ($n = 6$), for sausage types A and R, respectively. After storage, pH values were from 5.60 ± 0.00 and 5.43 ± 0.06 for sausage types A and R, respectively, and water activity remained at the same values.

Microbial analyses were performed on triplicate samples. Each sample was prepared from three sausages. Twenty five grams were removed (approximately 8 g from each sausage) and mixed into 225 ml of Peptone water (Merck; 7228) after being cut in 10 mm pieces. Tenfold 0.85% sodium chloride dilutions were prepared (Merck, 6404).

For the enumeration/isolation of bacteria, 1 ml samples (0.1 ml for staphylococci) from the dilution tubes were inoculated onto non-selective and selective media (Matos, Jensen, Barreto, & Hojberg, 2006).

Eighteen isolates of the spoilage bacteria were identified, based on phenotypic (cell morphology and fermentation profile) and genotypic (16S rDNA sequencing) characters as outlined by Matos et al. (2006).

The near-full-length sequences of the identified isolates, Te1A (*Enterococcus faecium*), Te2R and Te3R (*Pediococcus acidilactici*), Te11R (*Clostridium botulinum*), Te13A (*Staphylococcus epidermidis*), Te14R (*Enterococcus faecalis*), Te16R (*Clostridium aminovalericum*), Te18A (*Enterococcus solitarius*), Te21R (*Bacillus licheniformis*), Te22R (*Bacillus subtilis*), Te28A (*Bacillus cereus*), Te29R (*Bacillus pumilus*), Te30A (*B. cereus*), Te52A (*Lactobacillus curvatus*), Te58R (*E. faecium*), Te62A (*C. bifermentans*), Te63R (*C. celerecrescens*), and Te71A (*B. cereus*), have been deposited in GenBank under accession numbers AY587776, AY587802, AY587803, AY587804, AY587778, AY587805, AY587806, AY587780, AY587808, AY587809, AY587783, AY587814, AY587784, AY587792, AY587827, AY587793, AY587830, and AY587795, respectively.

2.2. Growth rate determination: 96-microwell plates preparation and experimental procedure

Overnight cultures of the *P. acidilactici*, *L. curvatus* and *E. faecium* isolates were prepared in MRS (Merck, 0661) broth with 0.5 g L^{-1} agar (MRSa-broth). Isolates of bacilli were cultured in PC medium. Reinforced clostridial broth (RCM) with 0.005 g L^{-1} hemin was used for culturing isolates identified as *C. botulinum*, *C. celecrescens*, *C. bifermentans*, *S. epidermidis* and *E. faecalis*. The isolates identified as *E. solitarius* and *C. aminovalericum* were cultured in a pre-reduced peptone yeast glucose (PYG) medium as described by Holdeman, Cato, and Moore (1977). Medium pH was measured after autoclaving. Each strain was tested in triplicate. Bacillus isolates were tested in PC medium with pH .10 and incubation at 30 °C. *P. acidilactici*, *L. curvatus* and *E. faecium* isolates were tested in MRSa medium in pH 5.44 and incubated at 30 °C. *E. faecalis*, *S. epidermidis*, *C. botulinum*, *C. bifermentans* and *C. celerecrescens* isolates were tested in RCM in pH 5.72 and incubated at 37 °C. Isolates of *C. aminovalericum* and *E. solitarius* were tested in pre-reduced steril-

ised peptone yeast glucose (PYG) medium in pH 7.20 and incubated at 37 °C.

Stocks of the preservative solutions, PS (Sigma S1751), SB (Sigma B3375) and methyl *p*-hydroxybenzoate, MHB (Sigma H5501) were prepared in distilled water in concentrations appropriate to reach the final concentration in the microwells of 0.01%, 0.05%, 0.10%, 0.15% and 0.30% (w/v). The applied salt concentrations were selected based on several studies related with the residual levels of the preservatives (Eklund, 1983; El-Banna & Hurst, 1983; Matos et al., 2005a; Stecchini, Del Torre, Donda, & Maltini, 2000; Thomas, Wimpenny, & Davis, 1993; Tompkin, Christiansen, Shaparis, & Bolin, 1974).

From tenfold diluted overnight cultures, aliquots of 25 μl were dispensed into the wells of a 96-microwell plate containing 200 μl of medium and 25 μl of salt solution. Finally, the wells were overlaid with 100 μl of liquid paraffin (to avoid evaporation) to obtain a final volume of 350 μl in each well. For positive controls, salt solutions were replaced by sterile water and for negative controls the culture was replaced by a corresponding volume of medium. All ingredients were prepared anaerobically (CO_2 atmosphere) according to Holdeman et al. (1977). The 96-microwell plates containing RCM and PYG were prepared in an anaerobic cabinet (10% CO_2, 10% H_2, 80% N_2).

Growth curves of the bacterial batches in each well of the microplates were obtained by reading absorbance at 620 nm (A_{620}) every 15 min for 17 h on an EL 808 – Ultra Microplate Reader (Bio-Tek Instruments, Inc.,Winooski, VE). The specific growth rates, μ, were calculated as the slope of the linear part of the ln-plots, representing the exponential growth phase. Results are presented as mean values ± standard deviation ($n = 3$) of the growth rate, μ (h^{-1}). Negative controls were subtracted from each result and positive controls corresponded to results obtained with 0% of salt concentration.

2.3. Statistical methods

Computational work was performed using SPSS for Windows 11.5 Software Package (Lead Technologies, Inc.). Mean values were compared using ANOVA methodology (Two Way ANOVA). This procedure was performed after certification of the adjustment of the results to Gauss curve (Kolmogorov–Smirnov Test) and application of the Levene Median Test. Statistical significance of differences between means of growth rate, μ (h^{-1}) was determined by the Tukey's test. Significance was established at $p < 0.05$.

3. Results

Growth rates of the isolates Te2R and Te3R (*P. acidilactici*), Te52A (*L. curvatus*), Te58R (*E. faecium*) (Fig. 2), Te16R (*C. aminovalericum*) (Fig. 3) and Te13A (*S. epidermidis*) (Fig. 4) were not significantly affected by any of the three preservatives ($p > 0.05$).

Growth rates of the *B. cereus* isolates (Te71A, Te30A, and Te28A), isolate Te21R (*B. licheniformis*) and isolate Te29R (*B. pumilus*) decreased with addition of the three preservatives ($p < 0.05$), in particular with MHB (Fig. 1).

Sodium benzoate did not affect growth rates ($p > 0.05$) of *B. subtilis* (Te22R). Nevertheless, we observed a significantly decrease ($p < 0.05$) in μ with increasing concentrations of MHB up to 0.15% and also with addition of PS, (independently of the salt concentration) when compared with the positive control (Fig. 1). PS and SB had less influence on these bacterial growth rates than MHB in pH 7.10 (Fig. 1).

Growth rates of the isolates *E. faecalis*, Te14R and *E. faecium*, Te1A, decreased in the presence of the three preservatives ($p < 0.05$) and, *E. solitarius* Te18A growth rate decreased significantly with increasing concentrations of MHB (Fig. 2).

The growth rate of *C. celerecrescens* (Te63R), *C. botulinum* (Te11R) and *C. bifermentans* (Te62A) significantly decreased with PS, SB and MHB (Fig. 3). However, *C. aminovalericum* (Te16R) growth rate was not affected by any of the preservatives.

PS and SB had less influence on the bacterial growth rates than MHB. However, the growth rates of three isolates of *B. cereus*, as well as single isolates of *C. celerecrescens*, *C. botulinum*, *C. bifermentans*, *E. faecalis* and *E. faecium* (Te1A) were increasingly inhibited with increasing concentrations of PS and SB up to 0.3% (w/v). Independently of the salt concentration, PS and SB also inhibited growth of the *B. licheniformis* and *B. pumilus* isolates.

4. Discussion

The decrease on the growth rate of *B. cereus* isolates (Te71A, Te30A, and Te28A), isolate Te21R (*B. licheniformis*) and isolate Te29R (*B. pumilus*) with addition of the three preservatives ($p < 0.05$) (Fig. 1) has also been observed by Stecchini et al. (2000). They showed that *B. cereus* growth rate in BHI agar (30 °C, pH 7.20) was significantly reduced with 0.2% PS. Also, Eklund (1983) showed that both dissociated and undissociated sorbic acid have antimicrobial activity on *B. subtilis*, *B. cereus*, *E. coli*, *P. aeruginosa*, *S. aureus* and *C. albicans* at neutral pH.

At pH 7.1, PS had less influence on *B. subtilis* (Te22R) growth rates than MHB and SB did not have any affect on growth rate of this isolate (Fig. 1). This may be due to the fact that *p*-hydroxy benzoic acid esters do not dissociate, and consequently their antimicrobial action is independent of the pH value. In this respect they are superior to the organic acids (Lueck, 1980).

The more effective antimicrobial ability of MHB against *E. solitarius* in comparison to the other two species of enterococci (*E. faecalis*, Te14R and *E. faecium*, Te1A) (Fig. 2) could have been caused by the different growth conditions (different pH, 7.20 vs 5.70 and 5.40) and the different media (PYG vs RCM and MRS).

The absence of inhibitory effects on *C. aminovalericum* (Te16R) compared to *C. celerecrescens* (Te63R), *C. botulinum* (Te11R) and *C. bifermentans* (Te62A) (Fig. 3) could be related with the low growth rates observed with this isolate under the applied experimental conditions (different pH of the medium, 5.7 vs 7.2). Tompkin et al. (1974) showed that growth of *C. botulinum* and botulinal toxin production in cooked, uncured sausages with 0.1% (m/m) of PS, kept at 27 °C, was retarded.

Several studies (Sofos, Busta, & Allen, 1979a, 1979b, 1980; Sofos, Busta, Bhothipaska, & Allen, 1979c) have demonstrated that sorbic acid (0.2%) may inhibit *C. botulinum* spore germination (loss of heat resistance) in chicken, beef and pork frankfurter - type emulsions. However, the antibotulinal effect of sorbic acid was found to be pH dependent and started at pH values just below 6.0. In addition, Tanaka, Worley, Sheldon, and Goepfert (1977) reported that the antibotulinal properties of sorbate in ground pork was ineffective at pH 6.3 but showed a strong effect at pH 5.5.

This study also showed that growth inhibition seems to be proportional to MHB concentration. Lueck (1980) also reported that the bacteriostatic action of esters is somewhat higher than that of the PS and SB due to the phenolic OH group of the former. Studies on the mechanism of action (Eklund, 1980, 1985; Freese & Levin, 1978; Freese, Shen, & Galliers, 1973) suggest that the parabens act mainly by causing disorganisation of the microbial cell membrane and, at low (bacteriostatic) concentrations, parabens appear to cause energy uncoupling which inhibits the uptake of metabolites, a bacteriostatic effect, whilst at higher (bactericidal) concentrations a loss of the membrane semipermeability produces the bactericidal effect. Also Hansch, Coubeils, and Leo (1972) concluded that the activity of the membrane active antibacterials such

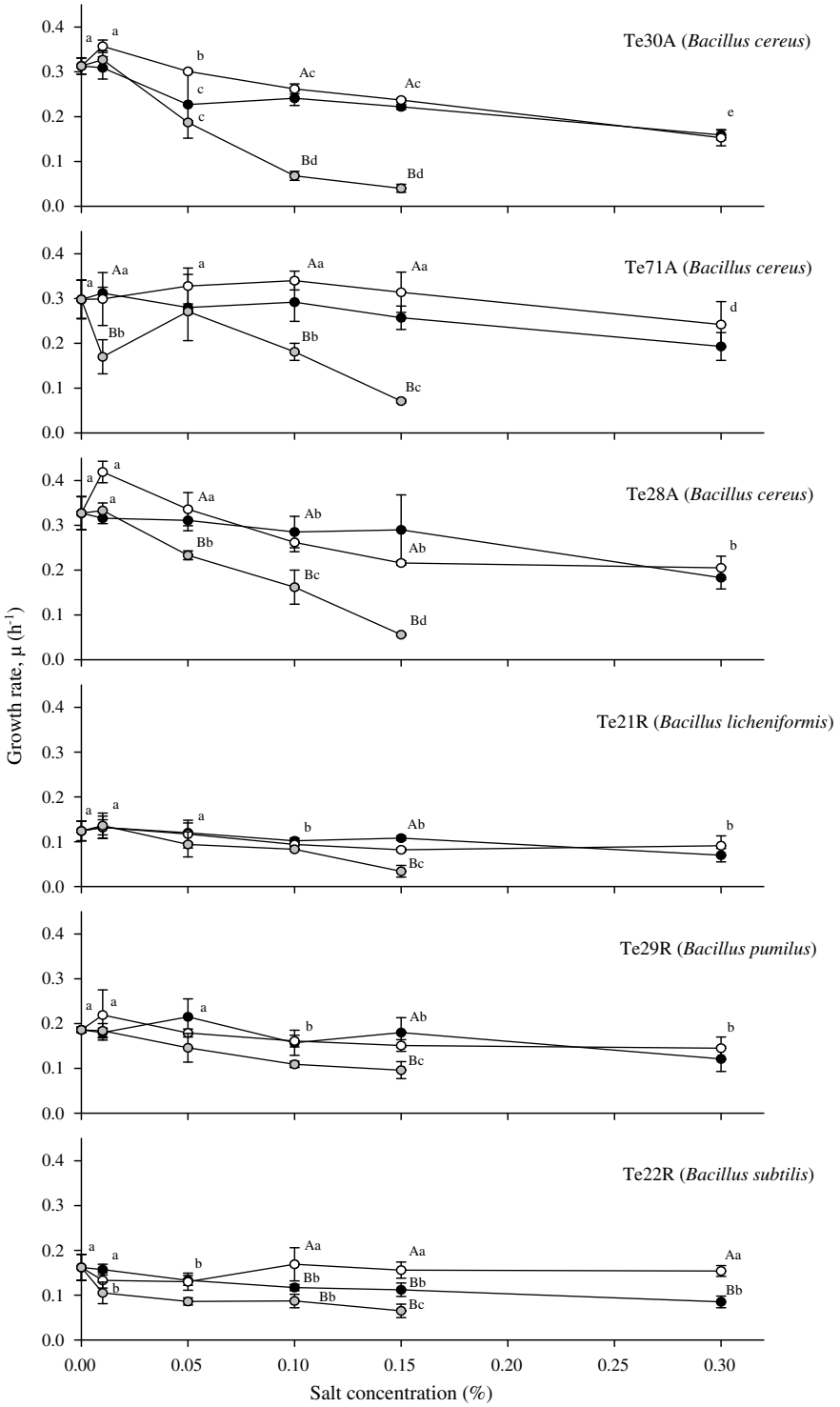

Fig. 1. Growth rates, μ (h^{-1}; mean values ± SD, $n = 3$) of representative *Bacillus* isolates recovered from *chouriço* type Alentejano (A) and Ribatejano (R) grown at 30 °C in microplates containing PC broth (pH 7.10). Potassium sorbate (black symbols) and sodium benzoate (white symbols) were added at five different concentrations and methyl *p*-hydroxybenzoate (grey symbols) at four different concentrations. For each salt, growth rates marked with different suffixes are significantly different ($p < 0.05$).

as the parabens depends on their ability to move freely in the aqueous phase, and yet be lipophilic enough to penetrate through the microbial outer cell envelope (where present) and the cytoplasmic membrane.

Regarding the huge economic losses (approx. 9% of the sales) as a consequence of product recalls (Matos, Barreto, & Bernardo, 2005b), and considering that surface treatments would represent less than 0.5% of the production costs of types of sausages used in this study (Matos et al., 2005a), MHB application as surface treatment could be considered as a relevant technological advantage to improve the stability and safety of the product through inhibition of potential spoilage and pathogenic microorganisms, such as sporeformers. However, investigations of the inhibitory effects of the preservative under more in situ studies would be necessary to provide information about the required inhibitory concentrations needed under industrial circumstances.

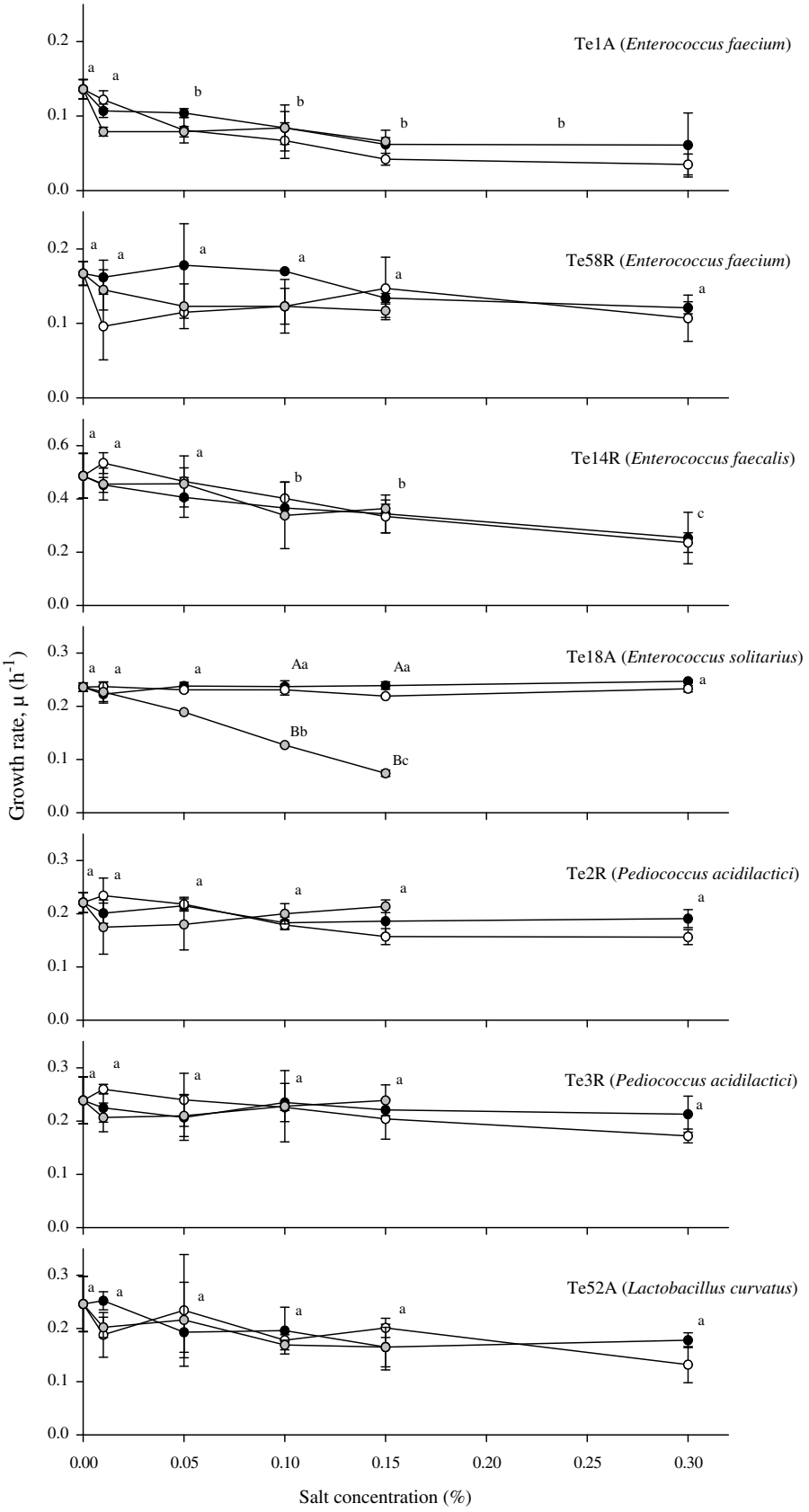

Fig. 2. Growth rates, μ (mean values ± SD, $n = 3$) of representative isolates of lactic acid bacteria recovered from *chouriço* type Alentejano (A) and Ribatejano (R). The isolates Te1A (*Enterococcus faecium*), Te2R (*Pediococcus acidilactici*), and Te52A (*Lactobacillus curvatus*) were grown in MRS broth (pH 5.4) at 30 °C, isolate Te14R (*E. faecalis*) in RCM broth (pH 5.7) at 37 °C, and isolate Te18A (*E. solitarius*) in PYG broth (pH 7.20) at 37 °C. Potassium sorbate (black symbols) and sodium benzoate (white symbols) were added at five different concentrations and of methyl *p*-hydroxybenzoate (grey symbols) at four different concentrations. For each salt, growth rates marked with different suffixes are significantly different ($p < 0.05$).

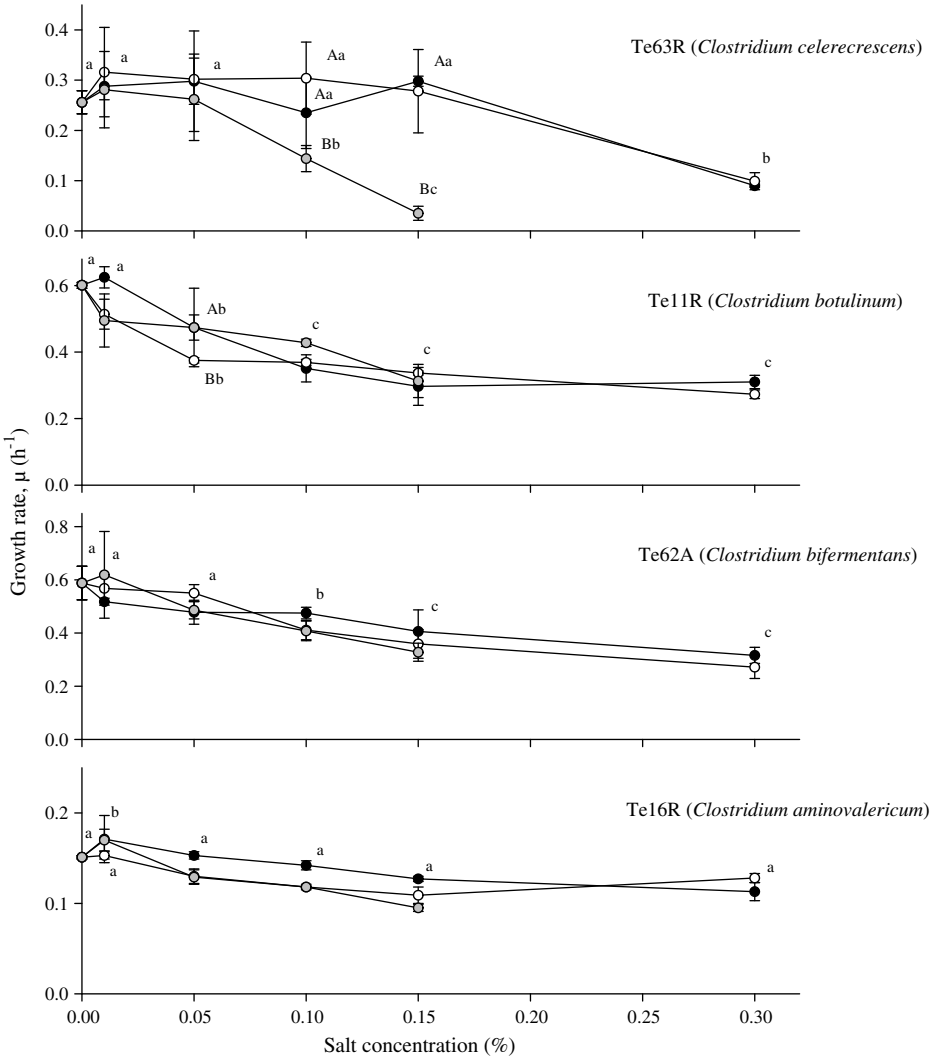

Fig. 3. Growth rates, μ (mean values ± SD, $n = 3$) of representative *Clostridium* isolates recovered from *chouriço* type Alentejano (A) and Ribatejano (R). Isolate Te16R (*C. aminovalericum*) was grown in PYG broth (pH 7.20) at 37 °C. The other isolates were grown in RCM broth (pH 5.7) at 37 °C. Potassium sorbate (black symbols) and sodium benzoate (white symbols) were added at five different concentrations and methyl *p*-hydroxybenzoate (grey symbols) at four different concentrations. Growth rate h^{-1} was determined at intervals of 15 min for 17 h (growth period). For each salt, growth rates marked with different suffixes are significantly different ($p < 0.05$).

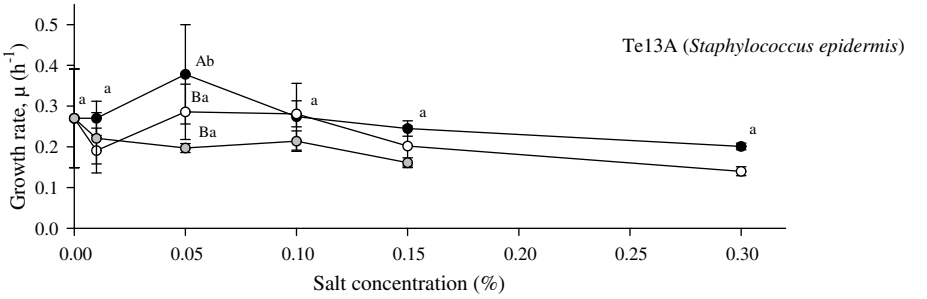

Fig. 4. Growth rates, μ (mean values ± SD, $n = 3$) of a *Staphylococcus* isolate recovered from *chouriço* type Alentejano (A). The isolate Te13A (*Staphylococcus epidermidis*) was grown in RCM broth (pH 5.7) at 37 °C. Potassium sorbate (black symbols) and sodium benzoate (white symbols) were added at five different concentrations and methyl *p*-hydroxybenzoate (grey symbols) at four different concentrations. For each salt, growth rates marked with different suffixes are significantly different ($p < 0.05$).

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