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Probiotic properties and fatty acid composition of the yeast *Kluyveromyces lactis* M3. *In vivo* immunomodulatory activities in gilthead seabream (*Sparus aurata*)

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ABSTRACT

The aim of this study was to analyze the probiotic potential, fatty acid composition and immunostimulant activities of *Kluyveromyces lactis* M3 isolated from a hypersaline sediment. For this purpose, *K. lactis* M3 resistance to different pH, salinities and bile, as well as its antioxidant capability were assayed. Furthermore, total fatty acid composition of the yeast was determined where the dominant fatty acids were palmitic, palmitoleic, oleic and linoleic acids. *K. lactis* M3 showed no cytotoxic effects on peripheral blood leukocytes. During an *in vivo* experiment in gilthead seabream (*Sparus aurata*), dietary *K. lactis* M3 supplemented at 0.55 or 1.1% of the basal diet enhanced bactericidal activity against *Vibrio parahaemolyticus* N16, *V. harveyi* Lg 16/00, and *V. anguillarum* CECT 43442 compared to fish fed commercial diet (control group). Finally, nitric oxide production, peroxidase activity and skin mucus lectin union levels strongly increased in fish fed *K. lactis* M3 with respect to the control group. The results suggested that the yeast *K. lactis* M3 had exhibited high antioxidant capability, and its dietary administration at 0.55 or 1% basal diet had immunostimulant activity for gilthead seabream. For all these reasons, it should be considered an appropriate probiotic candidate for the aquaculture fish industry.

1. Introduction

Currently, the interest on food additives with immunostimulant capability has been growing because of the promotion of "ecological health" free of antibiotics and synthetic drugs. Probiotics are live microorganisms that confer a health benefit on the host when administered in adequate amounts [1]. Numerous studies have highlighted that ideal probiotics should have several characteristics, such as (1) pH tolerance, (2) salt bile tolerance, (3) NaCl tolerance in case of marine fish, (4) high hydrophobicity and auto-aggregation capacities, (5) biosecurity and (6) high antioxidant capacity. All of them are properties with beneficial effects, including control of pathogens by competition for adhesion sites, antimicrobial compounds, and stimulation of the immune system [2–4]. Bacteria are commonly studied as probiotics although yeasts have gained much interest for their tolerance to several stressors (i.e. gastrointestinal tract conditions) and because they provide molecules, such as β-glucans, nucleotides, and polyamines that stimulate fish immune system [5,6]. Furthermore, renewed attention has been paid on non-*Saccharomyces* yeasts because several probiotic traits and bioactive compounds have beneficial attributes that sometimes are superior to *Saccharomyces cerevisiae* strains [7–9].

One of the most relevant non-*Saccharomyces* yeasts is *Kluyveromyces lactis*, which is an important yeast for research and industrial biotechnology because it has been generally recognized as safe (GRAS) status [10]. This yeast produces β-galactosidase, a native enzyme that catalyzes the hydrolysis of lactose to glucose and galactose, widely used in the dairy industry to generate lactose-free foods for individuals with lactose intolerance [11]. *K. lactis* is also used at industrial levels for the production of secondary metabolites and expression of recombinant proteins, including biopharmaceuticals [12]. Regarding probiotic attributes, *K. lactis* has gained attention in commercial field production because of its unique potential probiotic properties for animals and humans, such as an improved intestinal barrier function and enhanced immune functional activities [13–17]. Overall, those previous studies have demonstrated that *K. lactis* had very high adhesive ability and stimulated functional activities on immune cells, the production of

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immune-related molecules (receptors, immunoglobulins, and cytokines) and promoted antioxidant activities; which were mainly associated with yeast structural polysaccharides and soluble secreted molecules. However, there is a paucity of information regarding the effects of *K. lactis*, as well as other non-*Saccharomyces* yeasts, about its potential as probiotic or immunostimulant for aquatic animals in the aquaculture industry. While searching for non-*Saccharomyces* yeasts with probiotic potential for animal production, *Sterigmatomyces halophilus* strain N16 was recently isolated from extreme environments and evaluated in gilthead seabream (*S. aurata*) to enhance mucosal immunity against bacterial infection and be considered as a novel fish immunostimulant [1818]. The previous information is relevant because aquaculture industry has huge health problems caused by opportunist pathogens, such as *Vibrio* spp [19]. These bacteria mainly attach to skin and other external tissues [20,21]. Among all, *V. anguillarum*, *V. harveyi*, and *V. parahaemolyticus* have caused serious problems in gilthead seabream (*S. aurata*) rearing [18,19]. Therefore, *K. lactis* represents an alternative yeast to assess its probiotic properties against vibriosis in fish.

Because of the interest of the food industry in the selection of novel candidate probiotic strains, the probiotic potential of *K. lactis* M3 was evaluated for the first time for fish with a strain isolated from extreme environments and deposited at CIBNOR yeast collection. This study assessed *K. lactis* M3 for its microbiological properties (pH, salinity and bile resistance), antioxidant capability and fatty acid composition. Furthermore, the yeast was evaluated in peripheral blood leukocytes by using a cell viability assay. Finally, the *K. lactis* M3 capacity to modulate the innate immune system was determined in gilthead seabream by determining immune parameters and bactericidal activity in skin mucus.

2. Materials and methods

2.1. Yeast Kluyveromyces lactis M3

Kluyveromyces lactis M3 (Genebank accession number MK020404.1) was isolated from the sediment of a hypersaline marine environment (salt concentrator pond) located at the solar sea saltern "*Exportadora de Sal*, S.A. de C.V." (ESSA saltworks) in the Pacific coast of the Baja California Peninsula (Guerrero Negro, MX). *K. lactis* M3 was isolated in yeast peptone dextrose (YPD agar, Difco, Franklin Lakes, NJ, USA) medium containing 40 practical salinity units (psu = 4%) of NaCl at 30 °C for 24 h. Then, the yeast was removed with a bacteriological loop and suspended in YPD medium (100 mL or 1000 mL in Erlenmeyer flasks), followed by a new incubation on a rotary shaker (30 °C, 48 h) with constant aeration. The cell suspension was centrifuged (1000 × *g*, 4 °C, 5 min) and the pellet recovered. For probiotic abilities and antioxidant compound assays, *K. lactis* M3 was adjusted to a concentration of 1×10^9 cells mL^{-1} in phosphate buffer saline (PBS) according to Angulo et al. [22].

2.2. Bacterium

Bacteria used in this study were principal opportunistic pathogens in fish aquaculture, *V. parahaemolyticus* N16, *V. harveyi* Lg 16/00 *and V. anguillarum* CECT 43442.These bacteria were cultured in tryptic soy agar or tryptic soy broth (TSB, Difco, Franklin Lakes, NJ, USA) supplemented with NaCl to a final concentration of 1% (w/v) at 25 °C for 48 h. All bacteria were incubated at 28 °C for 24 h and then centrifuged at 8000 g, 4 °C for 20 min. Each bacterium was used at 1×10^8 colony forming units ml^{-1} for bactericidal activity.

2.3. Growth of Kluyveromyces lactis M3 at different pH

The growth of *K. lactis* M3 was assayed according to Park et al. [23]. First, yeast was inoculated (1:10 v/v) in liquid YPD medium and pH adjusted with 2 M HCl (2.5, 3.5, 4.5, and 5.5) and control (YPD, pH 6.5). The yeast cultures were incubated at 120 rpm, 30 °C for 30 min; then serial dilutions were performed (10^{-1}, 10^{-2}, and 10^{-3}). Finally, 100 µL of each dilution were inoculated in Petri dishes with YPD agar medium in triplicate at 30 °C for 24 h to assess viable unit forming colony (UFC mL^{-1}).

2.4. Resistance of Kluyveromyces lactis M3 at different concentrations of sodium chloride

For determination of NaCl tolerance, *K. lactis* M3 was grown in YPD broth medium in presence of NaCl (0, 1.5, 4.5 and 6.5%). The YPD broth was inoculated with 15 µL overnight culture of the isolates and incubated at 30 °C for 72 h. NaCl free YPD broth was used as control. Yeast growth was monitored by measuring absorbance at 600 nm (Varioskan 2.4.5, Thermo Scientific, Waltham, MA, USA), and unit forming colonies (ufc mL^{-1}) were calculated.

2.5. Gilthead seabream bile tolerance assay

The ability of *K. lactis* M3 to tolerate seabream bile salts was determined according to the modified method described by Gilliland et al. [24]. Briefly, YPD broth medium was supplemented with seabream (*S. aurata*) bile (50% of YPD medium, 40% of PBS, and 10% of bile). Subsequently, yeast was inoculated and incubated at 30 °C for 90 min [25]. Lastly, 100 µL of each dilution (10^{-1}, 10^{-2}, and 10^{-3}) were inoculated to assess viable colony counts (UFC mL^{-1}) in Petri dishes with YPD agar medium in triplicate at 30 °C for 24 h.

2.6. Cell surface hydrophobicity and auto-aggregation

A screening of hydrophobicity, assayed with a xylene test according to Brányik et al. [26], and auto-aggregation ability were performed in *K. lactis* M3. Briefly, *K. lactis* was cultured and centrifuged (8000 × *g*, 4 °C, 10 min). The supernatant was removed and cells washed with PBS. Finally, cells were resuspended and adjusted at 1×10^7 cells mL^{-1} in PBS, which corresponded to an absorbance of 0.5 at 600 nm. Then, xylene was added to the sample, allowing it to interact with cells at room temperature for 10 min. Thereafter, the aqueous phase was transferred in a 96-well microplate, and absorption (OD final) was read at 600 nm (Varioskan 2.4.5, Thermo Scientific, Waltham, MA, USA). The cell surface hydrophobicity of *K. lactis* M3 strains was calculated using the following formula:

Cell surface hydrophobicity (%) = (1- OD final/OD initial) x 100

The yeast aggregation test was conducted according to the method described by Kaushik et al. [27]. *K. lactis* M3 was harvested (8000 × *g* at 4 °C for 10 min), and the yeast pellet was washed twice. Cells were resuspended and adjusted at 1×10^5 cells mL^{-1} in PBS, which corresponded to an absorbance of 0.3 at 600 nm (Varioskan 2.4.5, Thermo Scientific, Waltham, MA, USA). Yeast suspension was incubated at 4 °C and collected after 4 and 24 h. The absorbance was measured at 600 nm (OD final), and yeast aggregation was calculated using the following formula:

Auto-aggregation (%) = (1- OD final/OD initial) x 100

2.7. Antioxidant capacities of Kluyveromyces lactis M3

2.7.1. ABTS radical scavenging activity

The 2,2′-Azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS, Sigma, St. Louis MO. USA) radical scavenging activity of *K. lactis* M3 was determined according to the method described by Ji et al. [28]. The $ABTS^{\cdot+}$ stock solution was produced by the reaction of ABTS aqueous solution (7 mM) with 2.45 mM aqueous

solution of potassium persulfate in equal quantities and allowing them to react at room temperature for 12–16 h in darkness until an absorbance of 0.700 at a 734 nm was reached (Varioskan 2.4.5, Thermo Scientific, Waltham, MA, USA). Then, 1 mL of $ABTS^{\cdot+}$ solution was mixed with 150 μL of intact yeast, incubated at 37 °C for 10 min and centrifuged at 8000 g, at 4 °C for 10 min. Ascorbic acid was used as standard and ABTS radical scavenging activity was calculated according to the following formula:

ABTS radical scavenging activity (%) = 1 - (OD sample/OD control) x 100

Where OD sample and OD control are the absorbance of each sample and distilled water, respectively, mixed with ABTS + solution.

2.7.2. Free radical scavenging activity

The reduction percentage rate of the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical by *K. lactis* M3 was performed according to Brand-Williams et al. [29]. This method is based on the reduction of a colored free radical DPPH− methanolic solution. The changes in color from deep-violet to light-yellow were measured at 515 nm in an ultraviolet (UV/visible) light spectrophotometer (Varioskan 2.4.5, Thermo Scientific, Waltham, MA, USA). Antioxidant activity was expressed as percentage according to the following formula:

DPPH radical scavenging activity (%) = 1 - (OD sample/OD control) x 100

Where OD sample and OD control are the absorbance of each sample and distilled water, respectively, mixed with DPPH solution.

2.7.3. Superoxide anion scavenging activity

The superoxide anion scavenging activity of *K. lactis* M3 was assayed according to the method reported by Martinez et al. [30]. The reaction mixture consisted of 50 mM sodium phosphate buffer (pH 7.8), 13 mM methionine, 100 μM EDTA, 75 μM NBT and 2 μM riboflavin. Fifty microliters of yeast were added to 3 mL of reaction mixture and incubated at 37 °C for 10 min; then, absorbance was measured at 560 nm (Varioskan 2.4.5, Thermo Scientific, Waltham, MA, USA). Butylated hydroxyanisole (BHA) was used as a positive control. The half maximal inhibitory concentration (IC50) value represented the concentration of yeast *K. lactis* M3 at which 50% of superoxide radicals were inhibited. The superoxide anion scavenging activity was measured according to the following formula:

Scavenging activity (%) = {(A0 − A1)/A0} × 100

Where A0 is the absorbance of the blank and A1 is the absorbance in the presence of the sample.

2.7.4. Determination of ferric-reducing antioxidant power (FRAP)

The ferric reducing ability of *K. lactis* M3 was measured according to Benzie and Strain [31]. Briefly, a working solution was prepared by mixing $FeCl_3$ solution (20 mM, 2.5 mL), TPTZ solution (10 mM 2,4,6-tripiridil-S-triazine in 40 mM HCl, 2.5 mL) and the acetate buffer (300 mM, 25 mL). Samples (1×10^8 cells mL^{-1}) of 75 μL were added to the working solution (1425 μL), and the mixture was incubated at 37 °C for 30 min. Absorbance was measured at 593 nm (Varioskan, Thermo Scientific, Waltham, MA, USA). Trolox was used as positive control, and dimethyl sulfoxide was negative control (Trolox and DMSO, Sigma, St. Louis MO USA). The results were expressed as percentage of ferric reducing antioxidant power.

2.8. Determination of the fatty acid profile of Kluyveromyces lactis M3

Total lipids were extracted from *K. lactis* M3 according to the method of Folch et al. [32] and methyl esters were generated by acid-catalyzed transesterification of total lipids according to Morrison and Smith [33]. The extraction was done with n-hexane, and the samples of methyl esters were separated by gas chromatography (GCMS-TQ8030, Duisburg, DE). The oven temperature of the gas chromatograph was programmed for 5 min at an initial temperature of 140 °C and increased at a rate of 3 °C min^{-1} to 230 °C; then, it was increased at a rate of 2 °C min^{-1} to 240 °C and held at that temperature for 12 min. The injector and flame ionization detector were set at 260 °C. Helium was used as the carrier gas at a pressure of 300 kPa, and peaks were identified by comparing their retention times with appropriate fatty acid methyl ester (FAME) standards (Sigma, St. Louis MO USA). Individual fatty acid concentration was expressed as percentage of the total content.

2.9. In vivo study: dietary administration of Kluyveromyces lactis M3 to gilthead seabream

Fifty-four gilthead seabream (5 ± 0.5 g mean body weight) specimens of the hermaphroditic protandrous seawater teleost gilthead seabream (*S. aurata* L.) were obtained from a local farm (Murcia, Spain). Fish were distributed at random and kept in six re-circulating seawater aquaria (50 L) in the Marine Fish Facility at the University of Murcia. Water temperature was maintained at 20 ± 2 °C with a flow rate of 900 L h^{-1} and 28‰ salinity. The photoperiod was 12 h light: 12 h dark and fish were fed with a commercial pellet diet (Skretting, Spain) at a rate of 2% body weight day^{-1}. Fish were allowed to acclimate for 7 days before the start of the experimental trial.

A standard commercial pellet diet for seabream (Skretting, Spain) was used as control diet and for being supplemented with two different concentrations of *K. lactis* M3: 0.55% and 1.1% (corresponding to 10^3 and 10^6 ufc per gram, respectively) [18], was performed as described in Garcia-Beltran et al. [34]. Three fish from each tank (n = 9) were randomly sampled at days 15 and 30 of the feeding trial. Fish were anesthetized prior to sampling with clove oil (100 mg L^{-1} marine water).

All applicable international, national, and institutional guidelines for the care and use of animals were followed. All experimental protocols were approved by the Ethical Committee of the University of Murcia (Permit number CEEA 357/2017).

2.10. K. lactis M3 cytotoxic activity on blood leukocytes

Blood samples were collected from the caudal vein with the aid of an insulin syringe from anesthetized specimens of *S. aurata*. Fish leukocytes were obtained, and cell viability was assayed according to that reported by Inoue et al. [35] with slight adjustments. In general, a 3.3-dihexyloxacarbocyanine (500 μg mL^{-1} ethanol) solution ($DiOC_6$, Sigma, St. Louis, MO, USA) was used. Then, $DiOC_6$ preparation was diluted (1:10) in Hank's balanced salt solution (HBSS, Sigma, St. Louis, MO, USA). Ten microliters of blood were mixed with HBSS (1950 μL), and 40 μL $DiOC_6$ solution were added. The mixture was incubated in darkness at 25 °C for 0.5 h for flow cytometric examination (FACSCalibur system, Becton Dickinson, San Jose, CA, USA). Analyses were performed by side scatter (SS) and forward scatter (FS) characteristics to gate blast cell populations.

2.11. Mucus immunological assays

Skin mucus samples were collected from anesthetized specimens according to the methodology described by Guardiola et al. [36].

2.11.1. Bactericidal activity

A colorimetric assay was developed for quantitating skin mucus bactericidal activity against *V. parahaemolyticus* N16, *V. harveyi* Lg16/00 *and V. anguillarum* CECT 43442 by MTT method [37]. Fish mucus samples of 20 μL were added to 96-well plates. PBS was used as positive control. Vibrio cultures (20 μL) were distributed into wells, and microplates were incubated at 25 °C for 5 h. Thereafter, 25 μL

(1 mg mL^{-1}) of thiazolyl blue tetrazolium bromide (MTT, Sigma, St. Louis MO USA) were dispensed and microplates incubated at 25 °C for 10 min. After centrifugation (2000 g, 10 min), 200 µL of DMSO were added. Finally, absorbance was recorded at 570 nm (Varioskan, Thermo Scientific, Waltham, MA, USA). The bactericidal activity was the difference between absorbance of living bacteria and positive controls (100%) and expressed in percentage.

2.11.2. Nitric oxide production (NO)

Nitric oxide production of skin mucus of gilthead seabream supplemented with *K. lactis* M3 was determined according to Neumann et al. [38]. Briefly, 100 µL of mucus was incubated with an equal volume of Griess reagent (Sigma, St. Louis MO USA) in a 96-well plate. The cells were incubated at room temperature in darkness for 15 min. At the end of incubation time, OD was read at 562 nm in a microplate reader (Varioskan, Thermo Scientific, Waltham, MA, USA). The data were expressed in nitrite concentration (µM).

2.11.3. Peroxidase activity

Total peroxidase (MPO) activity in mucus samples was measured according to Quade and Roth [39] with slight modifications. Optical density (OD) was read at 450 nm in a microplate reader (Varioskan, Thermo Scientific, Waltham, MA, USA).

2.11.4. Specific concanavalin A lectin binding levels

Specific lectin binding to mucus was determined by ELISA (enzyme linked immunosorbent assay, lectin ELISA). Briefly, skin mucus was dissolved 1:10 in 50 mM carbonate-bicarbonate buffer (pH 9.6), placed in flat-bottomed 96-well plates in triplicate and coated at 4 °C overnight. Microplates were washed with PBS (Sigma, St. Louis MO USA) containing 0.05% Tween 20 (PBT, pH 7.3) and then incubated with blocking buffer (3% BSA in PBT) at room temperature for 2 h. After washing, plates were incubated with 2 µg per well of biotinylated lectin Concanavalin A (ConA; with affinity for α-D-mannose and α-D-glucose, Sigma, St. Louis MO USA) for 1 h. Then, plates were washed and incubated with streptavidin-HRP (1:1000; Life Technologies) for 1 h. Finally, after an exhaustive washing with PBS-T, plates were developed using 100 µL of a 0.42 mM solution of 3,3′,5,5′-tetramethylbenzidine (TMB, Sigma, St. Louis MO USA) prepared in Milli-Q water containing 0.01% H_2O_2. The reaction was allowed for 10 min, stopped by the addition of 50 µL of 2 M H_2SO_4 and the plates were read at 450 nm in a microplate reader (Varioskan, Thermo Scientific, Waltham, MA, USA). Negative controls consisted of samples without skin mucus or without lectin, whose optical density (OD) values were subtracted from each sample value.

2.12. Statistical analysis

All bioassays and measurements were performed in triplicate and the mean ± standard deviation (SD) was calculated for each group and sampled time. A one-way analysis of variance (ANOVA) was performed to determine the effects of yeast *K. lactis* M3 on probiotic and immunological parameters using SPSS v.21.0 software (SPSS, Richmond, VA, USA). Means were separated by Tukey's multiple range test. Statistical analyses were made with the obtained data for each sample. Differences were considered statistically significant when $P < 0.05$.

3. Results

3.1. Kluyveromyces lactis M3 probiotic potential for fish

Kluyveromyces lactis M3 had similar growth in YPD medium at pH of 4.5 and 5.5 compared to control (pH 6.5). In contrast, its growth was affected ($p < 0.05$) at pH of 2.5 and 3.5 (Fig. 1a). *K. lactis* M3 growth in YPD medium was similar to control when YPD was supplemented with 1.5% NaCl but growth was statistically reduced ($p < 0.05$) when the yeast was cultured in YPD with 4.5 or 6.5% NaCl (Fig. 1b). In contrast, *K. lactis* M3 growth in YPD with gilthead seabream bile (10%) was unaffected compared to control samples (without bile) (Fig. 1c). In addition, *K. lactis* M3 had high cell surface hydrophobicity (80.6 ± 0.08%) and auto-aggregation (47.18 ± 5.52 and 87.30 ± 0.99%, at 4 and 24 h, respectively) properties.

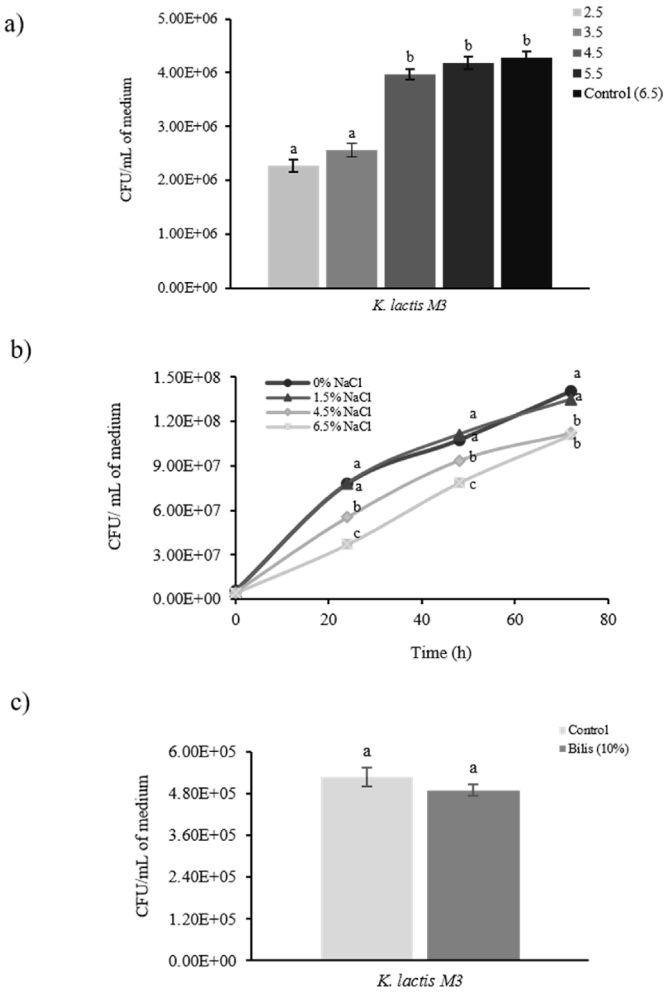

Fig. 1. Probiotic characteristics of *Kluyveromyces lactis* M3 cultured in yeast peptone dextrose (YPD) medium at 29 °C. (a) Growth of *K. lactis* M3 in YPD adjusted to different pH (control, 2.5, 3.5, 4.5, 6.5); (b) Growth curve of *K. lactis* M3 containing different concentrations of NaCl (0%, 1.5%, 4.5% and 6.5%) for 12, 24 and 48 h; (c) Growth curve of *K. lactis* M3 in YPD without (control) or containing 10% gilthead seabream bile. Each point and bar represent the mean ± SD. Different letters indicate significant ($p < 0.05$) difference among groups.

3.2. Kluyveromyces lactis M3 antioxidant capacity

Antioxidant capacity of *K. lactis* M3 is displayed in Fig. 2. As expected, ABTS, DPPH, and superoxide anion radical scavenging activities were lower in *K. lactis* M3 compared with pure antioxidant controls (Fig. 2 a,b,c). Remarkably, ferric reducing power was similar between *K. lactis* M3 and positive pure antioxidant control (Fig. 2d).

3.3. Kluyveromyces lactis M3 fatty acid profile

The fatty acid profile of the tested yeast was determined. *K. lactis* M3 had high content of palmitic (16:0), palmitoleic (16:1n-7), oleic (18:1n-9), linoleic (18:2n-6) and α-linoleic (18:3n-3) fatty acids (Table 1). Overall, the highest fatty acid group was monounsaturated

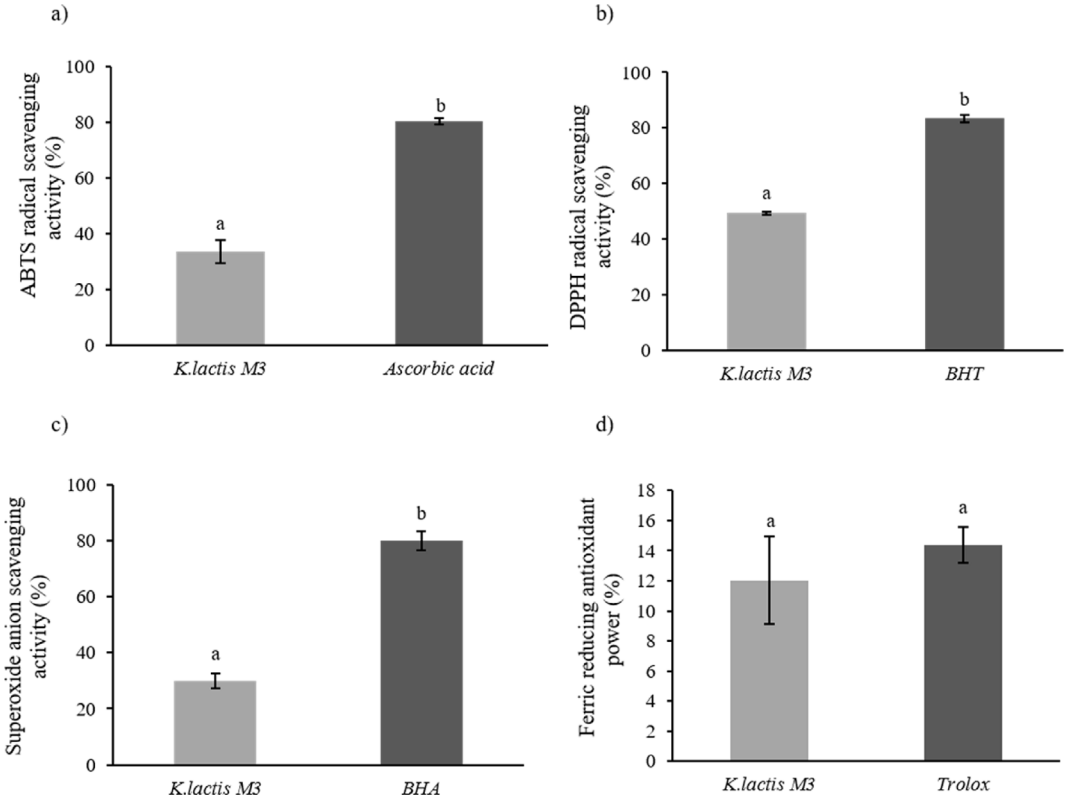

Fig. 2. Antioxidant activity of *Kluyveromyce lactis* M3 measured by (a) ABTS [2,2′-Azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt] radical scavenging activity; (b) DPPH (1,1-diphenyl-2-picrylhydrazyl) radical scavenging activity; BHT, Butylated hydroxytoluene; (c) superoxide anion scavenging activity; BHA, Butylated hydroxyanisole; and (d) Ferric reducing activity power; Trolox, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid. Bars represent the mean ± SD. Different letters indicate significant ($p < 0.05$) difference among groups.

(69.99%) followed by polyunsaturated n-6 (15.58%).

3.4. *Kluyveromyces lactis M3 is not cytotoxic for fish leucocytes*

Cell viability was analyzed in peripheral blood leucocytes of gilthead seabream (*S. aurata*) fed *K. lactis*. The results demonstrated that leukocyte viability was similar in fish fed *K. lactis* M3 at 0.55 or 1.1% diet compared with the control group (Fig. 3).

3.5. *Kluyveromyces lactis M3 enhanced mucus bactericidal activity and immune parameters*

Skin mucus of gilthead seabream fed *K. lactis* M3 had higher bactericidal activity against *Vibrio parahaemolyticus* N16, *V. harveyi* Lg 16/00, and *V. anguillarum* CECT 43442 compared to the control group (Fig. 4). Bactericidal activity in skin mucus against the three tested pathogenic bacteria was significantly higher ($p < 0.05$) in seabream fed *K. lactis* M3 at 0.55% or 1.1% level for 15 days. Curiously, the bactericidal activity found in skin mucus of fish fed 1.1% level for 30 days was statistically higher ($p < 0.05$) than those recorded in fish fed control diet or 0.55% *K. lactis* M3 diet (Fig. 4).

Table 1
Fatty acid composition (percentage, %) of *Kluyveromyces lactis* M3.

	%		%		%		%
14:0	0.415 ± 0.108	**14:1n-5**	0.159 ± 0.044	**18:2n-6**	15.559 ± 0.297	**18:3n-3**	1.500 ± 0.037
15:0	0.131 ± 0.024	**15:1n-5**	0.000 ± 0.000	**18:3n-6**	0.018 ± 0.018	**18:4n-3**	0.000 ± 0.000
16:0	9.594 ± 0.534	**16:1n-7**	34.214 ± 0.065	**20:2n-6**	0.000 ± 0.000	**20:3n-3**	0.021 ± 0.021
18:0	2.290 ± 0.113	**18:1n-9**	33.354 ± 0.502	**20:3n-6**	0.000 ± 0.000	**20:5n-3**	0.041 ± 0.041
20:0	0.179 ± 0.008	**18:1n-7**	0.981 ± 0.058	**20:4n-6**	0.000 ± 0.000	**22:5n-3**	0.000 ± 0.000
22:0	0.250 ± 0.019	**20:1n-9**	0.277 ± 0.014	**22:2n-6**	0.000 ± 0.000	**22:6n-3**	0.000 ± 0.000
		22:1n-9	0.000 ± 0.000	**22:4n-6**	0.000 ± 0.000		
		24:1n-9	1.009 ± 0.210				
Total saturated	12.862 ± 0.777	**Total monounsaturated**	69.996 ± 0.523	**Total n-6 PUFA**	15.5780 ± .285	**Total n-3 PUFA**	1.563 ± 0.034
Total PUFA (%)	17.141 ± 0.284						
n-3/n-6	0.100 ± 0.003						
Lipid (%)[a]	34.40 ± 0.2						

Mean ± SD (standard deviation) of triplicate determinations.
[a] Percentage of total lipids with respect to *Kluyveromyces lactis* M3 biomass.

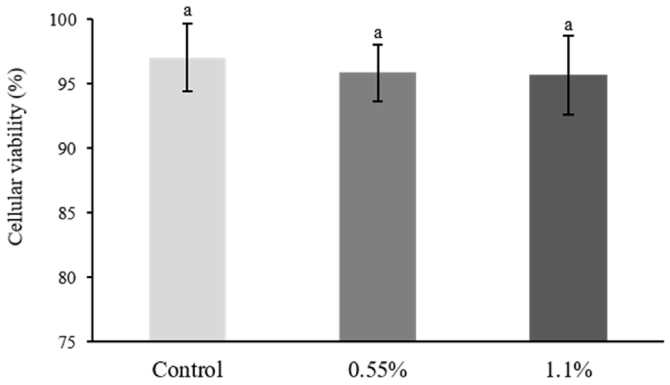

Fig. 3. Peripheral blood leucocyte viability of gilthead seabream fed *K. lactis* M3 after 30 days. Cell viability was determined by flow cytometry using $DiOC_6$. Bars represent the mean ± SD. Different letters indicate significant ($p < 0.05$) difference among groups.

Nitric oxide (NO) production in skin mucus of gilthead seabream increased in fish fed enriched diets, but the increments were statistically significant only in mucus from fish fed *K. lactis* M3 0.55% and 1.1% enriched diet for 15 and 30 days, respectively (Fig. 5a). Similar results were obtained for peroxidase activity and Concanavalin A lectin union. In both cases, the levels of this enzyme and the ConA union in skin mucus of fish fed *K. lactis* diets were always higher than those recorded in skin mucus of fish fed control diets. However, the increments were statistically significant only in skin mucus of fish fed *K. lactis* 0.55% diet (Fig. 5b and c).

4. Discussion

Probiotics based on yeast are widely used as animal feed supplements or as immunomodulatory additives. *K. lactis* is generally regarded as safe and has been studied for a long time as a host for heterologous protein expression [40,41]. These biological and biotechnological properties make *K. lactis* an excellent probiotic or immunostimulant candidate for animal production. Preliminary *in vitro* screening is essential to select a candidate yeast as probiotic. For this key reason, in this study, the main microbiological parameters used for screening potential probiotics were assayed for the selected yeast strain. *K. lactis* M3 was capable of resisting alive in culture media adjusted to pH of 4.5–6.5; it was able to grow at salinity up to 1.5%, and its growth was not affected by the presence of 10% gilthead seabream bile. The yeast also showed a high hydrophobicity (> 80%) and a strong self-aggregation capability (> 87%). All together, these attributes indicated that *K. lactis* M3 had a good potential colonization ability in the intestinal epithelial environment. The health-improving properties described for probiotics are mostly associated with a good adherence and subsequent colonization in the intestinal tract [42]. On the other hand, tolerance to fish bile is a very important characteristic for a probiotic, which must be able to grow and survive in the fish intestine [43], the place where the bile from liver is secreted. Fadda et al. [15] demonstrated that different strains of *K. lactis* have diverse probiotic abilities, showing that *K. lactis* KEFYRKL1 had the highest ability to survive in gastrointestinal conditions and bile salts.

In addition, hydrophobicity and auto-aggregation features are implicated in the adhesion capacity of probiotics and exclusion of pathogens at the intestine [44]. In the first case, cellular hydrophobicity was a result of protein and polysaccharide contents on the cellular surface, where major hydrophobicity indicated that major Lewis acid-basic interactions took place, which were present in protein-protein connections [45]. On the other hand, cell surface hydrophobicity may affect auto-aggregation ability (interaction between probiotic cells), thus the adhesion and colonization capacities of probiotics to epithelial cells and mucosal surfaces [46]. The highest hydrophobicity and self-

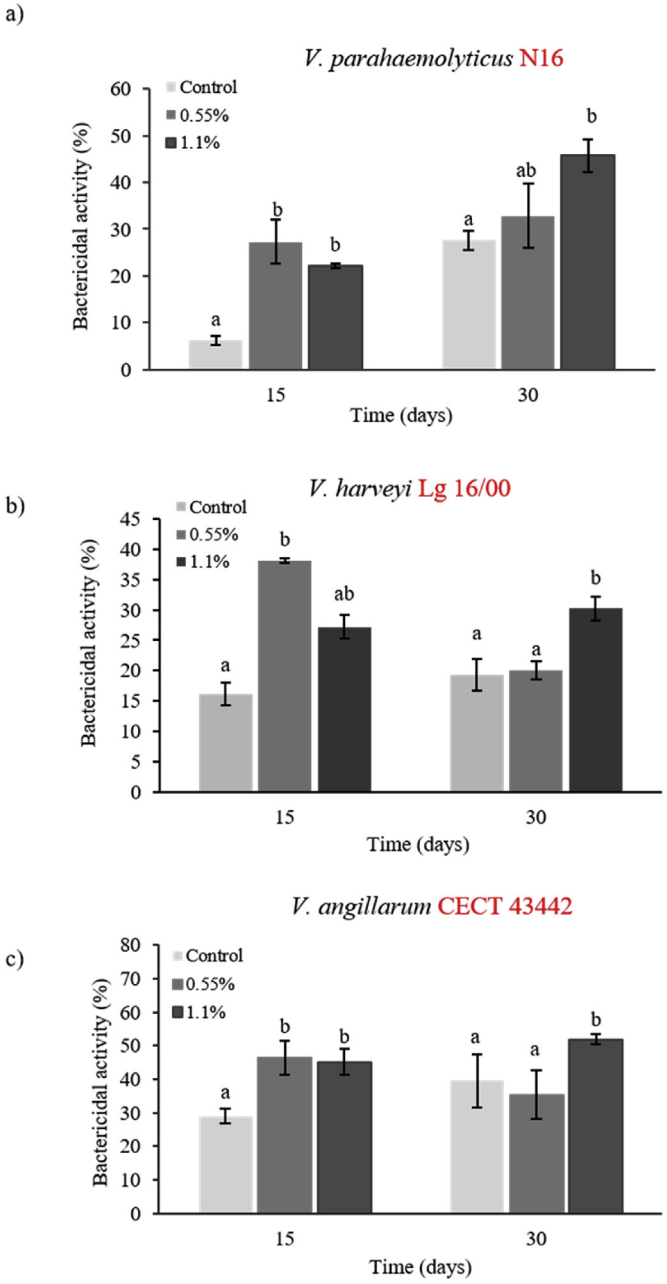

Fig. 4. Gilthead seabream bactericidal activity (expressed as percentage) determined in skin mucus of control fish (fed commercial diet) and fish fed supplemented with 0.5% and 1.1%*K. lactis* M3 for 15 and 30 days. (a) *Vibrio parahaemolyticus* N16; (b) *V. harveyi* Lg 16/00; and (c) *V. anguillarum* CECT 43442. Bars represent the mean ± SD. Different letters indicate significant ($p < 0.05$) differences among groups.

aggregation capacities of several *K. lactis* strains were 59 and 79%, respectively, which were lower values than those obtained for *K. lactis* M3 in this study. In line with those findings, other *K. lactis* strains also with potential probiotic features have been reported [13,16,17]. The available results seem to indicate that the yeast *K. lactis* is a promising probiotic to be considered in fish aquaculture.

Nutritional profile of the yeast is another important characteristic related with the environmental conditions. The cytoplasmic yeast membrane is composed of glycol sphingolipids, ergosterol, proteins and phospholipids [47]. The principal fatty acids in yeast included palmitic (16:0), palmitoleic (16:1), stearic (18:0), oleic (18:1), linoleic (18:2) and linolenic (18:3) acids [48]. In this sense, *K. lactis* M3 had a high

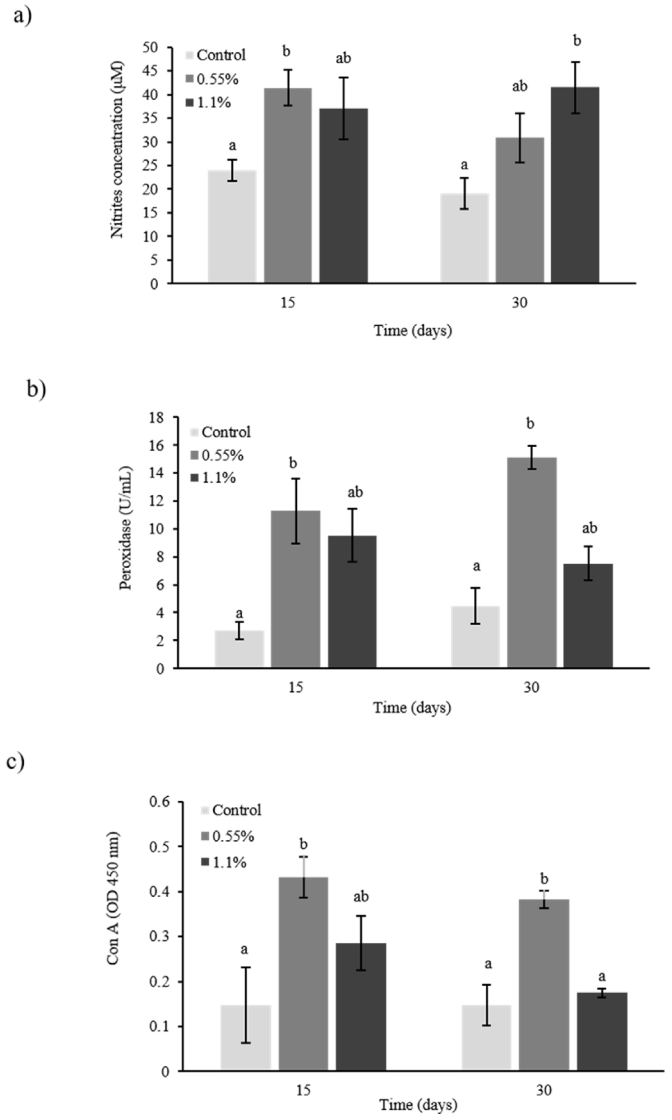

Fig. 5. Gilthead seabream skin mucus immune parameters of control fish (fed commercial diet) and fish fed supplemented with 0.5% and 1.1% *K. lactis* M3 for 15 and 30 days; (a) Nitric oxide production; (b) Peroxidase activity; and (c) Concanavalin A lectin union (OD 450 nm). Bars represent the mean ± SD. Different letters indicate significant ($p < 0.05$) differences among groups.

content of fatty acids and was mainly rich in monounsaturated or omega-9 fatty acids, such as palmitic, palmitoleic, oleic and linoleic fatty acids. Interestingly, *Kluveromyces marxianus*-fatty acid composition was related with its thermotolerance to high temperature [49], indicating that a species variability could occur on *K. lactis* M3 in response to stress induced by the technological conditions. In addition, probiotics promote antioxidant activity for prevention or reduction of oxidative damage caused by free radicals, nullifying the oxidative stress in the host [50]. This oxidative stress arises when antioxidants, such as catalase, superoxide dismutase and peroxidase enzymes are insufficient to neutralize the free oxygen radicals produced by the cells [51]. *K. lactis* M3 reported in this study showed a high antioxidant capacity, especially ferric reducing power (FRAP assay) when it was compared with a purified strong antioxidant. The antioxidant capacity of *K. lactis* M3 must rely on its structural (cell wall) or soluble (antioxidant enzymes) compounds able to donate electrons or hydrogen atoms to another molecule [52,53], thereby stabilizing free radicals and blocking radical chain reactions to the chemical oxidation of other molecules. In a previous study, Ceugniez et al. [54] observed a high antioxidant capacity of *K. marxianus* S-02-5 that also justified its evaluation as a good probiotic. Remarkably, the metabolic antioxidant capacity of probiotics may result from their own antioxidant enzymes, such as superoxide dismutase and catalase [55] or from the production of antioxidant metabolites [56]. More studies on enzyme and metabolic routes involved in antioxidant capacities could be useful to better understand the antioxidant abilities demonstrated in *K. lactis* M3.

On the other hand, the safety of a yeast is compulsory for its use as a probiotic in finfish. This study showed that *K. lactis* M3 was not cytotoxic for peripheral blood leukocytes. Our results agree with previous ones, for example Li et al. [57] reported that non-*Saccharomyces* yeasts were not toxic for intestinal Caco-2 cells (a continuous cell line obtained from a heterogeneous human epithelial colorectal adenocarcinoma) after 24 h of joint incubation. Similar results were obtained for *K. lactis* S-3-05 [54]. Likewise, the immunostimulant and immuno protective effects of *K. lactis* and *K. marxianus* strains are key features to be considered [14,58,59]. In this study, gilthead seabream feeding with dietary *K. lactis* M3 for 15 and 30 days had enhanced bactericidal activity in skin mucus. One of the most important first barrier in fish is the skin mucosal immune system, which comprises immune related enzymes that help in pathogen control [60]. Furthermore, *K. lactis* can also exclude some pathogens by producing different antimicrobial compounds [15]. The relevance of the probiotic properties found in this study indicated that *K. lactis* M3 induced in fish fed this yeast a high bactericidal activity in skin mucus against three pathogenic vibrio species: *V. parahaemolyticus* N16, *V. harveyi* Lg 16/00 and *V. anguillarum* CECT 43442. Interestingly, the specific lectin binding levels in skin mucus of fish fed the yeast significantly increased (0.5%) with respect to the values recorded for skin mucus of control fish (fed commercial diet). Lectins are carbohydrate-binding proteins found intra- and extracellularly in the skin mucus of several animal species, especially in fish. The function of animal lectins varies widely because they are involved in pathogen recognition, agglutination, opsonization, complement activation and phagocytosis [61]. Therefore, the observed increments in the lectin union to the skin mucus of gilthead seabream could be related with the high bactericidal activity also obtained in fish fed with *K. lactis* M3. The results in this study agree with other previous studies which have also confirmed that dietary probiotic yeasts can enhance skin mucus bactericidal activity in fish (i.e. Reyes-Becerril et al. [18]).

Yeast with probiotics properties can promote the activation of macrophages and other immune cells as part of their immunostimulant activity, which also consists of producing cytokines and inducing enzymes related to immune activity [62]. Immunological parameters are important to assess fish wellbeing and health status. Phagocytic activity induces the production of reactive oxygen species and enzymes, such as nitric oxide and peroxidases, respectively. Nitric oxide (NO) is an antimicrobial agent against pathogens [63], and myeloperoxidase activity is crucial for the generation of bactericidal HOCl from H_2O_2, enhancing mucosal immunity and keeping the redox balance [64]. In this study, higher levels of nitric oxide and peroxidase activity were observed as a result of the administration of dietary yeast *K. lactis* M3. Nitric oxide is a principal compound generated from the cellular metabolism of L-arginine, which is implicated in multiple metabolic and immunological pathways during *Vibrio* spp. infections [65–67]. On this regard and similar to our results using a probiotic yeast in gilthead seabream, the dietary administration of a bacterial probiotic increased NO production in skin mucus of tilapia *Oreochromis mossambicus* [68]. On the other hand, Maccaferri et al. [58] reported that *K. marxianus* stimulated the immune response in *in vitro* experiments using Caco-2 cells and peripheral blood mononuclear cells from humans. In human dendritic cells, *K. marxianus* modulated immune responses which may be very useful to control excessive inflammation [59]. In an *in vivo* experiment in chickens, Wang et al. [56] showed that *K. marxianus* increased quadratically serum lysozyme and IgG levels at doses of 1.0 g/kg. Interestingly, Kourelis et al. [14] found that *K. lactis* 630 induced the

recruitment of polymorphonuclear cells, phagocytic activity, and cytokine production in an air pouch rodent model. Moreover, mice fed *K. lactis* also had mucosal IgA and TLR expression in small intestine. Although any *K. lactis* strain has been previously studied as probiotic for fish, it is clear that oral delivery of *K. lactis* M3 could be regarded as an alternative approach for combating bacterial diseases and increasing humoral immune parameters in farmed gilthead seabream.

To conclude, the marine extremophile yeast *Kluyveromyces lactis* M3 seemed to be a very valuable source in terms of new promising probiotic with antioxidant capability and rich in monounsaturated fatty acid composition protecting from extreme conditions. Dietary *K. lactis* M3 also displayed an inhibitory effect against important pathogenic fish bacteria and enhanced the humoral immune parameters in skin mucus of gilthead seabream, an important marine fish species.

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