Agriculture and Food

AIMS Agriculture and Food, 4(2): 399–413.
DOI: 10.3934/agrfood.2019.2.399
Received: 31 December 2018
Accepted: 10 May 2019
Published: 24 May 2019

http://www.aimspress.com/journal/agriculture

Research article

Microbiological characterization of different formulations of *alheiras* (fermented sausages)

Julieta Silva[1], Joana Barbosa[1], Helena Albano[1], Maria Sequeira[1], Ana Pinto[1], Conceição Costa Bonito[2], Margarida Saraiva[2] and Paula Teixeira[1,*]

[1] Universidade Católica Portuguesa, CBQF—Centro de Biotecnologia e Química Fina—Laboratório Associado, Escola Superior de Biotecnologia, Rua Diogo Botelho 1327, 4169-005 Porto, Portugal
[2] INSA—Instituto Nacional de Saúde Doutor Ricardo Jorge, Rua de Alexandre Herculano 321, 4000-053 Porto, Portugal

* **Correspondence:** Email: pcteixeira@porto.ucp.pt; Tel: +351225580001; Fax: +351225090351.

Abstract: Different ingredients in old recipes are becoming popular and the traditional *alheira* did not escape to this new trend. The objective of this preliminary study was to characterize microbiologically nine different formulations of *alheira* from five producers. In this sense, isolates obtained were characterized through different phenotypic and biochemical tests. Their susceptibility to different antimicrobials and the presence of virulence factors was also investigated. Lactic acid bacteria were the predominant microbiota, but pathogenic bacteria as coagulase-positive staphylococci, *Listeria monocytogenes* and *Salmonella* spp. as well as indicator organisms were also found. Several virulence factors were produced among the different groups of isolates, with a high incidence of isolates producing β-haemolysis. Along with their potential pathogenic activity, also several antimicrobial resistances were found being the majority of isolates classified as multi-resistant. At our knowledge, this is the first study with these new formulations of *alheira*. A higher number of products must be analyzed, but we believe that results obtained in this study should help to alert consumers for the need of safe cooking time/temperatures of these products.

Keywords: *alheira*; antimicrobial resistance; virulence factors

Abbreviations: ATCC: American Type Culture Collection; aw: water activity; BEAA: Bile Esculin Azide Agar; BHI: Brain Heart Infusion; BPA: Baird Parker Agar; BPW: Buffered Peptone Water; CLSI: Clinical Laboratory Standards International; CFU: Colony Forming Unit; EFSA: European

Food Safety Authority; ISO: International Organization for Standardization; LAB: lactic acid bacteria; MIC: Minimum inhibitory concentrations; MLB: Modified Luria-Bertani; MRS: Man, Rogosa and Sharpe; MHA: Muller-Hinton Agar; PCA: Plate Count Agar; SXT: Trimethoprim/Sulfamethoxazole; TBX: Triptone Bile X-glucuronide; TPGY: Trypticase-Peptone-Glucose-Yeast Extract Broth; TSB: Tryptic Soy Broth; VRBD: Violet Red Bile Dextrose

1. Introduction

Fermented meat products are important elements in the economy of certain regions, not only because of their culinary heritage, but also due to their unique flavors and textures [1]. In Portugal, there are a wide variety of fermented meat products and their manufacture represents an important income in specific regions, predominantly in the North and the Southern regions [2]. Many of these products are fashionable food products that command high prices in urban centers and in export markets.

A traditional and naturally fermented meat sausage, typical from Trás-os-Montes region of northern Portugal is *alheira*. Traditionally, *alheira* is produced from chopped pork and poultry meat, lard, wheat bread, olive oil and pork fat, which are mixed with salt, garlic and spices. The meat, lard, olive oil and spices are boiled together with water and then the bread is added and the mass mixed. When everything is completely mixed the paste is stuffed into cellulose or natural pig casings and submitted to a smoking process for no longer than 8 days [3]. Nowadays, due to consumers and market demands, an increase of new products has been observed. Since 2015 that several formulations of *alheira* have been produced, both with different meats as turkey, piglet, lamb or veal, as well as with completely different ingredients like tuna, olives, between others.

Different formulations of traditional products, new ingredients in old recipes are trending. It is therefore necessary to characterize these new products in order to uphold their food safety and create the science base on which strategies for competitiveness and sustainability can be built. Such knowledge would allow the development of new methods of production and quality control, simultaneously compatible with modern retail channels and respectful of the unique characteristics of these products.

This preliminary study aimed to analyze the microbiota of nine different formulations of *alheiras*. The characterization of isolates through different phenotypic and biochemical tests as well as evaluation of their susceptibility to different antimicrobials and the presence of virulence factors was also performed.

2. Materials and methods

2.1. Sampling

Nine *alheiras* with different compositions and from five different producers were purchased from retail stores in two different occasions during October 2015 (referred as batch 1) and April 2016 (referred as batch 2). The innovative ingredients, pH and water activity of each *alheira* are presented in Table 1. Samples were transported to the laboratory in insulated bags and stored at 4 ℃ until they were analyzed.

Table 1. Innovative ingredients and intrinsic factors of nine formulations of *alheiras*.

Alheira	Producer	Main different ingredient	Batch	pH	a_w
1	A	Tuna fish (50%) and thyme	1	5.54	0.995
			2	5.37	0.998
2		Chicken meat (30%), pitted olive (25%) and oregano	1	5.43	0.981
			2	5.19	0.993
3		Red wine	1	5.60	0.982
			2	5.22	0.993
4	B	Chicken meat (40%) and brown bread (30%)	1	3.92	0.993
			2	4.43	0.989
5		Turkey meat (40%)	1	4.56	0.991
			2	4.97	0.988
6	C	Lamb meat (30%)	1	3.84	0.978
			2	4.18	0.968
7	D	Veal meat (35%)	1	4.73	0.983
			2	5.56	0.983
8	E	Corn bread	1	4.95	0.987
			2	5.31	0.975
9		Piglet meat (45%)	1	5.34	0.979
			2	5.21	0.983

Legend: a_w—water activity at 23 ±1 ℃ (Aqualab, Series 3, Decagon Devices Inc., Pullman, WA, USA).

2.2. Microbiological analyses

Several pieces of each *alheira*, randomly selected until a total of 25 g, were added to 225 mL of sterile buffered peptone water (BPW, Merck, Darmstadt, Germany) and homogenized in a stomacher for 2 min. Appropriate decimal dilutions were prepared in sterile Ringer's solution (LabM, Bury, UK) for microbial enumeration according to ISO Standards: total microorganisms at 30 ℃ on plate count agar (PCA, Pronadisa, Madrid, Spain; [4]), lactic acid bacteria on de Man, Rogosa and Sharpe Agar (MRS, Biokar Diagnostics, Beauvais, France; [5]) and enterococci on Bile esculin azide agar (BEAA, Biokar Diagnostics; [6]); *Enterobacteriaceae* on violet red bile dextrose agar (VRBD, Merck; [7]) and coagulase-positive *staphylococci* on Baird Parker Agar (BPA, Pronadisa; ISO [8]); *Escherichia coli* on Triptone Bile X-glucuronide Agar (TBX, Bio-Rad, CA, USA; ISO [9]) and yeasts and moulds on Rose-Bengal Chloramphenicol Agar (Oxoid, Hampshire, UK; [10]). The detection of some agents was also performed: *Listeria monocytogenes* on pre-enrichment Half Fraser Broth (Merck; [11]); *Salmonella* spp. on pre-enrichment BPW [12] and sulfite-reducing *Clostridium* spores according to NP 2262: 1986 [13]. For each parameter evaluated, two independent analyses were performed using randomly selected pieces.

After appropriate incubation, colonies were counted and/or confirmatory tests performed and the colony forming units (CFU)/g calculated. Microbial counts were transformed to log CFU/g.

2.3. Statistical analysis

An analysis of variance was carried out to test the differences between each formulation of *alheira* as well as between different producers. Multiple comparisons were evaluated by Tukey's

post-hoc test and all analyses were performed using IBM SPSS Statistics, 24 (IBM Corporation, USA). The mean difference was considered significant at the 0.05 level.

2.4. Origin of isolates

Colonies grown on each selective media were randomly selected and purified by repeated streaking onto the respective growth media. All isolates (10%) recovered were stored at −80 ℃ in Tryptic Soy Broth (TSB, Pronadisa) or MRS broth (in the case of LAB) with 30% (v/v) of glycerol (Sigma, Steinheim, Germany) and sub-cultured twice before use.

2.5. Characterization of isolates

2.5.1. Identification by phenotypic and biochemical tests

Isolates were characterized using their colonial and cellular morphology, Gram reaction and conventional biochemical tests: enterococci (n = 38) were tested for different growth conditions and acid production from several sugars [14]; *Listeria* spp. isolates (n = 7) were identified according to the ISO 11290-1: 1996 [11]; staphylococci (n = 49) were tested for the presence of several enzymes, acid production from several sugars [15] and susceptibility to novobiocin (5 μg) and polymyxin B (300 IU) according to Iorio et al. [16]. *Enterobacteriaceae* (n = 33) were tested for motility, production of hydrogen sulfide, presence of several enzymes and acid production from several sugars [15]. Lactic acid bacteria (LAB, n = 111) were tested by Gram staining and for the presence of enzymes catalase and oxidase [5].

Identification of each group of isolates was based on Bergey's Manual of Determinative Bacteriology [15].

2.5.2. Screening for botulinum neurotoxin producing strains

Samples which showed the presence of sulfite-reducing *Clostridium* were screened for botulinum neurotoxin producing strains by mouse bioassay [17] following enrichment in TPGY (Trypticase-Peptone-Glucose-Yeast Extract Broth).

2.5.3. Antimicrobial susceptibility

The minimum inhibitory concentrations (MIC; μg/ml) were determined by ε-test for trimethoprim/sulfamethoxazole (SXT, AB Biodisk, Solna, Sweden) and by the agar dilution method for fifteen other antimicrobials, according to the Clinical Laboratory Standards International [18]. Each test was carried out on Muller-Hinton Agar (MHA, BioMérieux) with cations adjusted for penicillin G (Sigma) and ampicillin (Fluka, Steinheim, Germany) and on MHA for vancomycin (Fluka), ceftazidime, chloramphenicol, kanamycin, nalidixic acid, nitrofurantoin, oxacillin, streptomycin (Sigma), ciprofloxacin, erythromycin, gentamicin, tetracycline and rifampicin (all kindly supplied by the company Labesfal, Portugal). Each test was carried out on Muller-Hinton Agar, using a different set of antimicrobials for each group of microorganisms [18,19]. All the isolates were grown on plates of MHA and MHA with cations adjusted with no antimicrobial. Each

experiment was performed in duplicate and the quality control strains *Enterococcus faecalis* ATCC 29212 and *Staphylococcus aureus* ATCC 29213 were used to monitor the accuracy of MICs [18]. Plates were incubated at 37 ℃ for 24 hours.

Enterococcus spp., *Staphylococcus* spp. and *Enterobacteriaceae* isolates were classified as sensitive, intermediate or resistant to each antimicrobial according to the Clinical and Laboratory Standards Institute [18]. Lactic acid bacteria (LAB) and *Listeria* spp. isolates were classified as described by EFSA [19] and Barbosa et al. [20], respectively.

Isolates exhibiting resistance to, at least, two of the antimicrobial agents of different classes were considered to be multi-resistant strains.

2.5.4. Presence of virulence factors

Presence of different virulence factors was only determined for *Staphylococcus* spp., LAB isolates, *Enterococcus* spp. and *Enterobacteriaceae*.

1) Production of gelatinase, DNase and hemolytic activity

Presence of hydrolytic enzymes gelatinase [21] and DNase [22] was determined using the modified Luria-Bertani (MLB) broth supplemented with 50.0 g/L of gelatin and DNase agar (Pronadisa) with 0.05 g/L of methyl green (Sigma), respectively. Presence of haemolysin was assessed using 5% v/v Sheep blood agar [23]. Each experiment was performed in duplicate and *S. aureus* ATCC 25213 was used as positive control.

2) Detection of decarboxylase activity

Only isolates of LAB, *Enterococcus* spp. and *Enterobacteriaceae* were screened for the production of histamine, tyramine, putrescine and cadaverine, according to the method described by Bover-Cid and Holzapfel [24]. Briefly, each isolate was sub-cultured seven times in MRS (LAB) or Brain Heart Infusion broth (BHI; *Enterobacteriaceae* and *Enterococcus* spp.) with 0.1% of each precursor amino-acid (all from Sigma), in order to promote enzyme induction. Then, all isolates were spotted in duplicate on the Bover-Cid medium plates with and without (as control) each amino acid and incubated at 37 ℃ for 4 days under aerobic conditions. Positive reaction was confirmed when a purple color occurred or tyrosine precipitate disappeared around the colonies [24,25].

3. Results and discussion

Microbiological characteristics of nine *alheiras* with different formulations were studied in two different time periods. The innovative ingredients of each fermented sausage and their respective values of pH and water activity (a_w) are presented in Table 1. Values of pH varied between 3.84 and 5.60 and values of a_w between 0.968 and 0.998. Traditionally, besides displaying distinctive organoleptic and sensory characteristics in these products, low values of pH and a_w are also important parameters to prevent microbial spoilage and growth of pathogenic bacteria [26]. Combination of different hurdles is important to ensure the safety of these products. The use of

spices such as thyme or oregano (producer A) may contribute as additional hurdles, due to their recognized antimicrobial activities [27].

Regarding composition of the selected fermented sausages, only one did not include meat (*alheira* 1, tuna fish), four were produced with different meats, such as turkey (*alheira* 5), lamb (*alheira* 6), veal (*alheira* 7) and piglet (*alheira* 9), two were produced with chicken meat and uncommon ingredients such as brown bread instead of wheat bread (*alheira* 4) and pitted olive and oregano (*alheira* 2) and, finally, despite their traditional composition corn bread and red wine were added to *alheiras* 8 and 3, respectively.

Results for the enumeration and detection of different microorganisms are presented in Table 2 and statistical differences obtained between formulations of *alheiras* and producers are shown in supplementary Tables S1 and S2. Differences in manufacturing processes and/or composition of *alheiras* could be the reason of the great variability between different producers as well as different products and different time of production from the same producer. With the exception of *alheiras* from producer A, no significant differences were obtained between the other producers ($p > 0.05$), since all presented high values of total microorganisms at 30 ℃. Counts in MRS were also high for the majority of the samples, which was expectable since *alheiras* are fermented products and LAB play an important role in fermentation processes [28]. Also *Enterococcus* spp. were present in most of the samples and, when present, their values varied between 2.8 and 8.7 log CFU/g. Although a lot of benefits have been attributed to their presence in fermented products, such as contribution on ripening and aroma development [29] and also the production of antimicrobial substances [25], the existence of many strains possessing virulence factors and which are becoming increasingly resistant to antimicrobials is a reason of concern [23,30,31]. Apart from producer E, in which none sample presented yeasts or moulds, in the other samples, higher growth was observed for yeasts compared to moulds. It is described that these microorganisms also play an important role in the development of the organoleptic characteristics of products [32].

The presence of indicator microorganisms was also found in a few samples, which might be result of poor hygiene or poor process control [26]. *Enterobacteriaceae* were found *in alheiras* from three producers (A, D and E), but counts were lower than 4 log CFU/g in all samples and in *alheira* 8 (batch 2) of producer E, *E. coli* was found in numbers of 2.3 log CFU/g. Also three samples from batch 1 (*alheiras* 1, 4 and 7) and three samples from batch 2 (*alheiras* 2, 5 and 8) were positive for indicator organism *Listeria* spp. and, in addition, the pathogen *L. monocytogenes* was present in three samples (from batch 1: *alheira* 4 and from batch 2: *alheiras* 2 and 5). Although this pathogen was present in only one *alheira* of producer A, the remaining two positive *alheiras*, although having different compositions, belong to producer B. This may be indicative of cross-contamination during their manufacture [33]. Although *alheiras* are cooked before consumption, it is important to highlight that cooking methods might not be sufficient to inactivate this foodborne pathogen [34].

Table 2. Microbial characterization of nine different formulations of *alheiras*.

			Enumeration (log CFU/g)								Presence in 25g			
Alheira	Producer	Batch	Total microorganisms at 30 ℃	Counts in MRS	Enterococci	Yeasts	Moulds	*Enterobacteriaceae*	*E. coli*	Coagulase positive staphylococci	*Listeria* spp.	*Listeria monocytogenes*	*Salmonella* spp.	SRC spores[a]
1	A	1	6.43 ±0.04	6.23 ±0.04	3.95 ±0.24	5.59 ±0.05	1.81 ±0.05	3.05 ±0.08	<1.00 ±0.00	1.30 ±0.00	+	−	−	(−)1g
		2	>5.48 ±0.00	3.28 ±0.07	2.77 ±0.10	2.64 ±0.02	<1.00 ±0.00	<1.00 ±0.00	<1.00 ±0.00	<1.00 ±0.00	−	−	−	(−)1g
2		1	6.88 ±0.03	6.70 ±0.03	6.68 ±0.08	4.08 ±0.05	2.67 ±0.05	<1.00 ±0.00	<1.00 ±0.00	1.45 ±0.21	−	−	+	(−)1g
		2	4.52 ±0.03	4.65 ±0.12	<2.00 ±0.00	3.23 ±0.05	<1.00 ±0.00	<1.00 ±0.00	<1.00 ±0.00	2.31 ±0.08	+	+	−	(−)1g
3		1	2.81 ±0.03	2.31 ±0.10	<2.00 ±0.00	2.25 ±0.07	<1.00 ±0.00	<1.00 ±0.00	<1.00 ±0.00	<1.00 ±0.00	−	−	−	(−)1g
		2	5.54 ±0.04	5.44 ±0.02	<2.00 ±0.00	2.54 ±0.04	<1.00 ±0.00	<1.00 ±0.00	<1.00 ±0.00	<1.00 ±0.00	−	−	−	(−)1g
4	B	1	>9.48 ±0.00*	3.85 ±0.09	3.32 ±0.06	2.57 ±0.11	2.00 ±0.00	<1.00 ±0.00	<1.00 ±0.00	1.48 ±0.00	+	+	−	(−)1g
		2	9.00 ±0.00	9.00 ±0.00	6.45 ±0.02	<1.00 ±0.00	<1.00 ±0.00	<1.00 ±0.00	<1.00 ±0.00	<1.00 ±0.00	−	−	−	(−)1g
5		1	8.55 ±0.05	8.66 ±0.04	7.80 ±0.03	<1.00 ±0.00	<1.00 ±0.00	<1.00 ±0.00	<1.00 ±0.00	<1.00 ±0.00	−	−	−	(−)1g
		2	8.48 ±0.04	8.31 ±0.09	7.95 ±0.06	<1.00 ±0.00	<1.00 ±0.00	<1.00 ±0.00	<1.00 ±0.00	<1.00 ±0.00	+	+	−	(−)1g
6	C	1	>9.48 ±0.00	8.98 ±0.04	8.64 ±0.01	1.77 ±0.10	2.17 ±0.24	<1.00 ±0.00	<1.00 ±0.00	4.41 ±0.04	−	−	−	(−)1g
		2	9.34 ±0.09	9.28 ±0.03	8.74 ±0.06	<1.00 ±0.00	<1.00 ±0.00	<1.00 ±0.00	<1.00 ±0.00	>5.18 ±0.02	−	−	−	(−)1g
7	D	1	>9.48 ±0.00	8.97 ±0.04	5.89 ±0.27	2.89 ±0.07	1.78 ±0.25	2.85 ±0.03	<1.00 ±0.00	2.50 ±0.16	+	−	−	(−)1g
		2	9.38 ±0.01	9.30 ±0.00	4.10 ±0.02	<1.00 ±0.00	<1.00 ±0.00	2.80 ±0.04	<1.00 ±0.00	2.70 ±0.02	−	−	−	(−)1g
8	E	1	>9.48 ±0.00	7.44 ±0.01	<2.00 ±0.00	<1.00 ±0.00	<1.00 ±0.00	<1.00 ±0.00	<1.00 ±0.00	<1.00 ±0.00	−	−	−	(+)1g/(−)0.1g
		2	8.90 ±0.00	8.74 ±0.00	4.45 ±0.04	<1.00 ±0.00	<1.00 ±0.00	1.81 ±0.05	2.30 ±0.03	4.20 ±0.04	+	−	−	(−)1g
9		1	8.11 ±0.17	8.00 ±0.07	5.84 ±0.10	<1.00 ±0.00	<1.00 ±0.00	<1.00 ±0.00	<1.00 ±0.00	<1.00 ±0.00	−	−	−	(+)1g/(−)0.1g
		2	8.40 ±0.00	2.29 ±0.00	3.88 ±0.03	<1.00 ±0.00	<1.00 ±0.00	<1.00 ±0.00	<1.00 ±0.00	1.06 ±0.03	−	−	−	(+)1g/(−)0.1g

Legend: [a]SRC spores; sulphite reducing clostridial spores, presence (+) or absence (−) in 1, 0.1 or 0.01 g sample; *The authors were not able to justify such high values for total counts and lower values for the other groups of microorganisms investigated.

Regarding pathogens, significant differences were obtained between formulations ($p < 0.05$) with three samples: one from batch 1 (*alheira* 7) and two from batch 2 (*alheiras* 2 and 7) presenting more than 2 log CFU/g of coagulase-positive *Staphylococcus* and other three: one from batch 1 (*alheira* 6) and two from batch 2 (*alheiras* 6 and 8) presenting more than 4 log CFU/g. These high concentrations are probably the result of the considerable levels of handling product-in-process or even cross-contamination [35]. *Salmonella* spp. was present in 25g of *alheira* 2 (batch 1) and sulfite-reducing *Clostridium* spores in 1 g of three samples (*alheira* 8 (batch 1) and *alheira* 9 (batches 1 and 2)). As in September 2015 there were cases of botulism associated with the consumption of *alheiras* in Portugal, positive samples for sulphite-reducing *Clostridium* spores were sent to *Instituto Ricardo Jorge* to evaluate their possible contamination by *Clostridium botulinum*. Neurotoxigenic strains of *C. botulinum* were not detected in the three positive samples. Although different, all samples belong to the same producer, which indicates that cross-contamination could occur during the production processes.

Only samples from producers A and B - *alheira* 1 (batch 2), *alheira* 3 (batches 1 and 2), *alheira* 4 (batch 2) and *alheira* 5 (batch 1) did not show the presence of any pathogen. It is interesting to note that from those, only *alheira* 3 had no pathogens in the two batches tested. Although very similar to traditional *alheiras*, the huge difference is that these samples were produced with red wine (wine marinade with added garlic), which may contribute to their good microbiological quality. Antimicrobial activities of wine marinades are already reported [36].

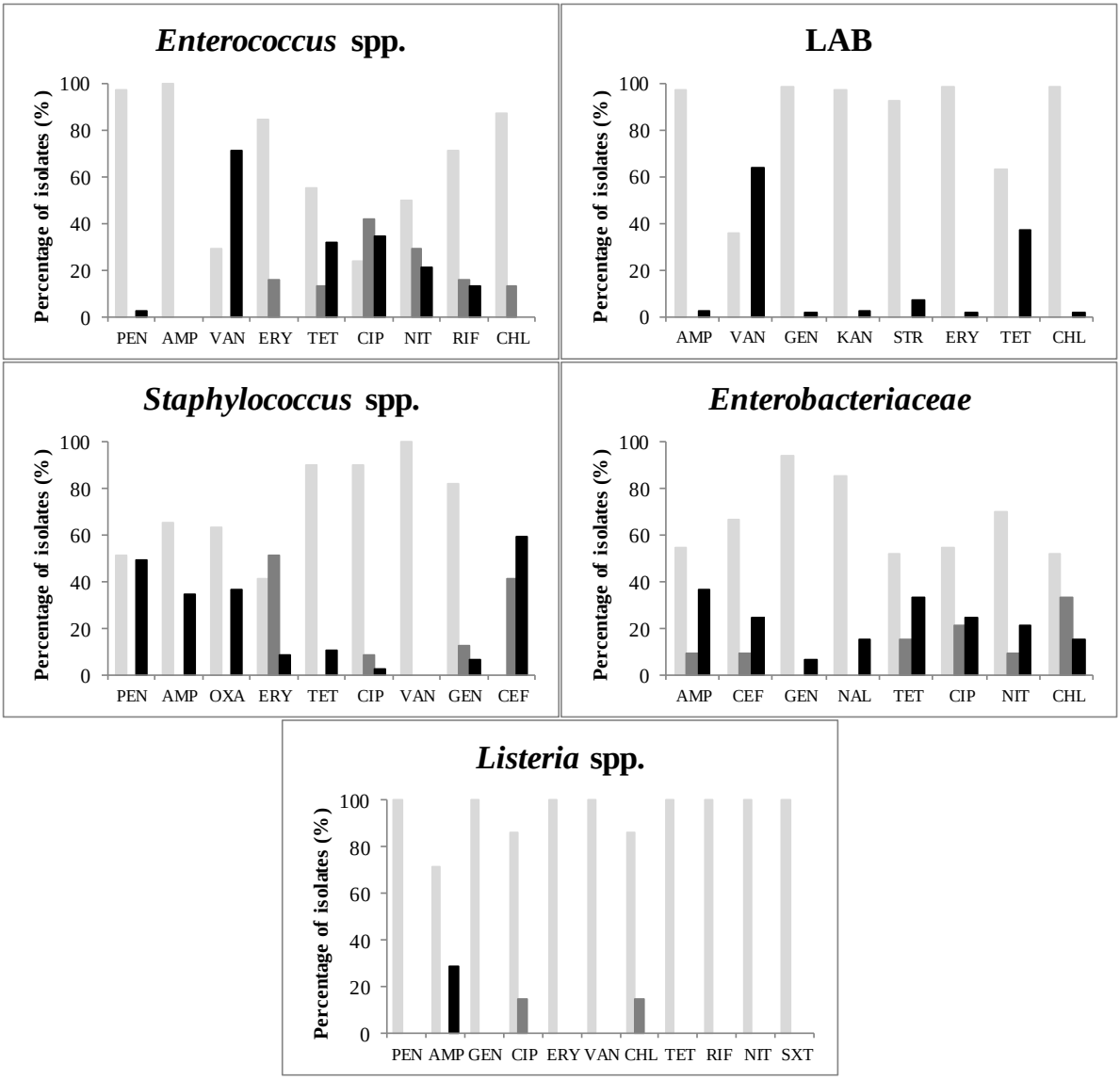
Results obtained by different phenotypic and/or biochemical tests (data not shown) only allowed the characterization and identification of LAB to group level, *Enterobacteriaceae* to family level, enterococci and staphylococci to the genus level, and *Listeria* spp. to species level (*L. monocytogenes* and *L. innocua*).

The percentage of isolates (belonging to each group of bacteria) that were sensitive, intermediate and resistant to each tested antimicrobial is presented in Figure 1.

All isolates of enterococci (n = 38) were susceptible to ampicillin (100%) and a large number to penicillin (97.37%), chloramphenicol (86.84%), erythromycin (84.21%) and rifampicin (71.05%). Those results are in agreement with the study of Barbosa et al. [30] for enterococci isolated from traditional fermented products, with the exception of erythromycin and rifampicin, since the authors found a high percentage of resistant isolates. The same authors also reported intermediate resistance of 0.5% to vancomycin, instead of 71.05% of resistance found in the current study. Apart from the high percentage of enterococci isolates resistant to vancomycin, also high percentages were found to ciprofloxacin (34.21% resistant and 42.11% intermediate resistant) followed by tetracycline (31.58% resistant and 13.16% intermediate resistant) and nitrofurantoin (21.05% resistant and 28.95% intermediate resistant). Furthermore, 30 out of 38 isolates were multi-resistant. Multi-resistances from enterococci isolated from other fermented meat products have been already reported by other authors [30,37]. One of the most important concerns regarding the presence of antimicrobial resistant enterococci is their remarkable ability to acquire new mechanisms of resistance and also to transfer resistance genes to other pathogens [38].

Regarding other isolates belonging to LAB group (n = 111), none of the isolates showed intermediate resistances, more than 90% of the isolates were sensitive to all antimicrobials, except to vancomycin (63.96% resistant) and tetracycline (63.06% resistant) and 31 out of 111 isolates were multi-resistant. Several species of *Lactobacillus*, *Leuconostoc* and *Pediococcus* are intrinsically resistant to vancomycin, which could explain the high percentages found [39]. In the study of Federici et al. [40], the authors also found resistances to tetracycline among isolates from different

genera of LAB isolated from smoked and dry-cured meat sausages. Unlike resistance to vancomycin, resistance to tetracycline is acquired and there is evidence of conjugative transfer of the gene *tet(M)* from lactobacilli to other microorganisms *in vitro* [41], which is a matter of concern.



Legend: PEN—penicillin G, AMP—ampicillin, VAN—vancomycin, ERY—erythromycin, TET—tetracycline, CIP—ciprofloxacin, NIT—nitrofurantoin, RIF—rifampicin, CHL—chloramphenicol, GEN—gentamicin, KAN—kanamycin, STR—streptomycin, OXA—oxacillin, CEF—ceftazidime, NAL—nalidixic acid, SXT—trimethoprim/sulfamethoxazole.

Figure 1. Percentage of isolates (%), belonging to different genera, family or group, that were sensitive (light grey bars), intermediate (dark grey bars) or resistant (black bars) to each set of antimicrobials.

More than 80% of coagulase-positive *Staphyloccus* spp. isolates (n = 49) were sensitive to tetracycline (89.80%), ciprofloxacin (89.80%) and gentamicin (81.63%) and all were sensitive to

vancomycin (100%). It is noteworthy the resistance of isolates to ceftazidime (59.18% resistant and 40.82% intermediate resistant), followed by penicillin (48.98% resistant), oxacillin (36.73% resistant), ampicillin (34.69% resistant) and erythromycin (8.16% resistant and 51.02% intermediate resistant) and, importantly, the multi-resistance of 41 out of 49 isolates. Studies reporting the prevalence of antimicrobial resistance among *Staphylococcus* isolated from fermented sausages are scarce. In the study of Marty et al. [42], less than 50% of coagulase-negative staphylococci isolated from spontaneously fermented meat products were resistant to the antimicrobials tested. Rebecchi et al. [43] found a high frequency of coagulase-negative staphylococci isolated from a typical Italian salami resistant to tetracycline and erythromycin. Pereira et al. [44] also studied 65 coagulase-positive staphylococci isolated from traditional Portuguese fermented meat products and despite the authors having found a higher percentage of penicillin and ampicillin resistant isolates, they also found percentages of isolates resistant to oxacillin, ciprofloxacin, erythromycin and vancomycin similar to those found in this study.

Concerning *Enterobacteriaceae* (n = 33), high percentages of sensitive isolates were found to gentamicin (93.94%) and nalidixic acid (84.85%), but more than 30% of the isolates were resistant to ampicillin (36.36%) and tetracycline (33.33%) and intermediate resistant to chloramphenicol (33.33%). In addition to all the resistances observed, 21 out of 33 isolates were multi-resistant. During recent years several studies have reported the antimicrobial resistance of some genera of *Enterobacteriaceae* isolated from meat, such as *Escherichia* spp. and *Salmonella* spp. [45]. The scarcity of studies with antimicrobial resistances of *Enterobacteriaceae* isolated from fermented products turns difficult the comparison of the results obtained in the present study. However, despite the large percentage of susceptible isolates, the presence of antimicrobial resistance genes that can be transmitted to other microorganisms and the ability of *Enterobacteriaceae* to acquire new resistances must be taken into account.

It is possible to observe that isolates of *Listeria* spp. (n = 7) were sensitive to most of the antimicrobials tested. Only resistance to ampicillin (28.6% resistant), ciprofloxacin (14.3% intermediate resistant) and chloramphenicol (14.3% intermediate resistant) were observed. Also none of the isolates were multi-resistant. It is important to highlight that only isolates of *L. innocua* were resistant to ampicillin. In a study with 121 *L. monocytogenes* isolated from *alheiras* and/or their raw materials, the authors also found a low incidence of antimicrobial-resistant isolates [20].

Phenotypic expression of virulence factors gelatinase, DNase, haemolysis and biogenic amines of each group of isolates are presented in Table 3.

Only 10.2% of staphylococci isolates produced gelatinase, but DNase production was detected in 36.7% of isolates and also 57.1% of the isolates showed β-haemolytic activity. In the study of Pereira et al. [44], the authors found higher incidence of staphylococci isolates producers of gelatinase and β-haemolysis. This could be explained due to the fact that isolates in this study whereas in the study of Pereira et al. [44] only *S. aureus* were investigated.

Incidence of virulence factors tested among LAB and enterococci isolates was similar. About 10% produced gelatinase and only 4 LAB isolates (3.6%) produced DNase, but higher incidences were observed for haemolysin production with almost 50% of isolates showing β-haemolysis (48.6% of LAB and 52.6% of enterococci). Some studies with fermented products have been demonstrating similar results in terms of production of gelatinase [23,31,46] and DNase [14,23] for enterococci and other LAB. However, the same is not valid for haemolysin production, since the same authors reported very low incidences (nearly 0%) of β-haemolytic activities. In fact, the protein toxin

cytolysin, which is responsible to β-haemolysis is the most important virulence factor recognized to enterococci genus [31] and the high incidence of β-haemolytic isolates found is a matter of concern.

Table 3. Phenotypically expression of virulence factors by isolates (%) from different formulations of *alheira*.

				Haemolytic activity			Biogenic amines			
Isolates	n	Gelatinase	Dnase	β	α	ɣ	Cadaverine	Histamine	Putrescine	Tyramine
Staphylococcus spp.	49	5 (10.2)	18 (36.7)	28 (57.1)	5 (10.2)	16 (32.7)	na	na	na	na
LAB	111	11 (9.9)	4 (3.6)	54 (48.6)	7 (6.3)	50 (45.0)	3 (2.7)	2 (1.8)	7 (6.3)	0 (0.0)
Enterococcus spp.	38	4 (10.5)	0 (0.0)	20 (52.6)	1 (2.6)	17 (44.7)	13 (34.2)	3 (7.9)	9 (23.7)	3 (7.9)
Enterobacteriaceae	33	9 (27.3)	5 (15.2)	2 (6.1)	1 (3.0)	30 (90.9)	0 (0.0)	0 (0.0)	0 (0.0)	8 (24.2)

In contrast, low incidences of haemolysin production were found among *Enterobacteriaceae* isolates (6.1% β-haemolysis and 3.0% α-haemolysis), but 27.3% and 15.2% of isolates produced gelatinase and DNase, respectively. Although being virulence factors, gelatinase and DNase are tests used in identification of some genera/species of *Enterobacteriaceae*. Therefore, to our knowledge, does not seem to exist data about the presence of these factors in *Enterobacteriaceae* isolated from fermented sausages. However, in a study with *Enterobacteriaceae* associated with ready-to-eat fruits, the authors found high incidences of gelatinase (81.7%) and α-haemolysin (96.6%) producers [47].

Positive reactions were also found for all biogenic amines in the screening medium for LAB and enterococci isolates, except for tyramine in the case of LAB isolates. It has been reported that tyramine is frequently the most abundant biogenic amine found in fermented sausages [48] and produced mainly by LAB, including enterococci [49]. Although the low incidence, 7.9% of enterococci isolates produced tyramine. For isolates of *Enterobacteriaceae* only positive reactions for tyramine were observed (24.2%). Durlu-Özkaya et al. [50] also reported the ability of *Enterobacteriaceae* isolated from meat to produce tyramine, as well as putrescine, cadaverine and histamine.

Usually, putrescine and cadaverine are found in fermented products in lower levels than tyramine but some authors have been demonstrating the ability of several LAB in producing those two biogenic amines [46,49,51]. Histamine is rarely found in fermented sausages and its production appears to be restricted to some strains of a small number of *Enterobacteriaceae* or LAB isolates (reviewed by [52]).

4. Conclusions

New formulations of *alheira* were microbiologically characterized. Although lactic acid bacteria were the predominant microbiota, pathogenic bacteria were also found. This means that the same microbiological hazards found in the traditional *alheira* were also found in the new formulations. This is not surprising, since the same producers are producing the new products and the possibility of cross-contamination is high. It is still necessary to alert to safe cooking time/temperatures of these products, since some common cooking practices of *alheira*, may not guarantee a sufficient temperature in its center to eliminate the pathogens potentially present.

Also several virulence factors were found among the different group of isolates tested, with a high incidence of isolates producing β-haemolysis. Along with their potential pathogenic activity, also several antimicrobial resistances were found being the majority of isolates classified as multi-

resistant. Given that these products provide a perfect environment for contact between bacteria, the easiness of horizontally transmission of antimicrobial resistance genes, or virulence determinants, is a matter of concern.

Even taking into account that a larger number of products must by analyzed, the results obtained in this study should not be ignored and, moreover, should serve to alert consumers for a correct preparation of these products.

Acknowledgments

This work was supported through project 'Biological tools for adding and defending value in key agro-food chains (bio—n2—value)', No. NORTE-01-0145-FEDER-000030, funded by *Fundo Europeu de Desenvolvimento Regional* (FEDER), under *Programa Operacional Regional do Norte—Norte2020*". We would also like to thank the scientific collaboration under the FCT project UID/Multi/50016/2019. Financial support for author J. Barbosa was provided by a post-doctoral fellowship SFRH/BPD/113303/2015 (FCT).

Conflict of interest

All authors declare no conflicts of interest in this paper.

References

1. Ojha KS, Kerry JP, Duffy G, et al. (2015) Technological advances for enhancing quality and safety of fermented meat products. *Trends Food Sci Technol* 44: 105–116.
2. Marcos C, Viegas C, De Almeida AM, et al. (2016) Portuguese traditional sausages: different types, nutritional composition, and novel trends. *J Ethnic Foods* 3: 51–60.
3. Albano H, Oliveira M, Aroso R, et al. (2007) Antilisterial activity of lactic acid bacteria isolated from 'Alheiras' (traditional Portuguese fermented sausages): *in situ* assays. *Meat Sci* 76: 796–800.
4. ISO 4833-1: 2013. Microbiology of the food chain - Horizontal method for the enumeration of microorganisms—Part 1: Colony count at 30 °C by the pour plate technique.
5. ISO 15214: 1998. Microbiologie des aliments—Méthode horizontale pour le enombrement des bactéries lactiques mésophiles—Technique par comptage des colonies à 30 °C.
6. Ferreira V, Barbosa J, Vendeiro S, et al. (2006) Chemical and microbiological characterization of *alheira*: A typical Portuguese fermented sausage with particular reference to factors relating to food safety. *Meat Sci* 73: 570–575.
7. ISO 21528-2: 2000. Microbiology of food and animal feeding stuffs - Horizontal method for the detection and enumeration of *Enterobacteriaceae*—Part 2: colony-count method.
8. ISO 6888-1: 1999. Microbiology of food and animal feeding stuffs—Horizontal method for the enumeration of coagulase-positive staphylococci (*Staphylococcus aureus* and other species)—Part 1: Technique using Baird-Parker agar medium.
9. ISO 16649-2: 2001. Microbiology of food and animal feeding stuffs—Horizontal method for the enumeration of beta-glucuronidase-positive *Escherichia coli*—Part 2: Colony-count technique at 44 degrees C using 5-bromo-4-chloro-3-indolyl beta-D-glucuronide.

10. NP 3277-1: 1987. Food Microbiology: Moulds and Yeast colony counting—Part 1: Incubation at 25 ℃.
11. ISO 11290-1: 1996. Microbiology of food and animal feeding stuffs - Horizontal method for the detection and enumeration of *Listeria monocytogenes* - Part 1: Detection method.
12. ISO 6579: 2002. Microbiology of food and animal feeding stuffs—Horizontal method for the detection of *Salmonella* spp..
13. NP 2262: 1986. IPQ food microbiology. General rules for identifying reducing Clostridium sulphite spores.
14. Albano H, van Reenen CA, Todorov SD, et al. (2009) Phenotypic and genetic heterogeneity of lactic acid bacteria isolated from 'Alheira', a traditional fermented sausage produced in Portugal. *Meat Sci* 82: 389–398.
15. Holt JG, Krieg NR, Sneath PHA, et al. (1994) Bergey's Manual of determinative bacteriology, 9th edition, Lippincott Williams & Wilkins.
16. Iorio NLP, Ferreira RBR, Schuenck RP, et al. (2007) Simplified and reliable scheme for species-level identification of *Staphylococcus* clinical isolates. *J Clin Microbiol* 45: 2564–2569.
17. CDC (1998) Botulism in the United States. Handbook for epidemiologists, clinicians, and laboratory workers. Centers for disease control and prevention. National center for infectious diseases. Division of bacterial and mycotic diseases.
18. Clinical and Laboratory Standards Institute (2012) Performance Standards for Antimicrobial Susceptibility Tests; Document M100. Wayne, PA: Clinical and Laboratory Standards Institute.
19. European Food Safety Authority (2012) Guidance on the assessment of bacterial susceptibility to antimicrobials of human and veterinary importance by EFSA Panel on Additives and Products or Substances used in Animal Feed (FEEDAP). *EFSA J* 10: 2740–2749.
20. Barbosa J, Magalh ães R, Santos S, et al. (2013) Evaluation of antibiotic resistance patterns of food and clinical *Listeria monocytogenes* isolates in Portugal. *Foodborne Pathog Dis* 10: 861–866.
21. Tiago I, Teixeira I, Silva S, et al. (2004) Metabolic and genetic diversity of mesophilic and thermophilic bacteria isolated from composted municipal sludge on poly-e-caprolactones. *Curr Microbiol* 49: 407–414.
22. Ben Omar N, Castro A, Lucas R, et al. (2004) Functional and safety aspects of enterococci isolated from different Spanish foods. *Syst Appl Microbiol* 27: 118–130.
23. Barbosa J, Gibbs PA, Teixeira P (2010) Virulence factors among enterococci isolated from traditional fermented meat products produced in the North of Portugal. *Food Control* 21: 651–656.
24. Bover-Cid S, Holzapfel WH (1999) Improved screening procedure for biogenic amine production by lactic acid bacteria. *Int J Food Microbiol* 53: 33–41.
25. Barbosa J, Borges S, Teixeira P (2014) Selection of potential probiotic *Enterococcus faecium* isolated from Portuguese fermented food. *Int J Food Microbiol* 191: 144–148.
26. Ferreira V, Barbosa J, Silva J, et al. (2007) Characterisation of *alheiras*, traditional sausages produced in the North of Portugal, with respect to their microbiological safety. *Food Control* 18: 436–440.
27. Govaris A, Botsoglou E, Sergelidis D, et al. (2011). Antibacterial activity of oregano and thyme essential oils against *Listeria monocytogenes* and *Escherichia coli* O157:H7 in feta cheese packaged under modified atmosphere. *LWT Food Sci Technol* 44: 1240–1244.
28. Zakpaa HD, Imbeah CM, Mak-Mensah EE (2009) Microbial characterization of fermented meat products on some selected markets in the Kumasi metropolis, Ghana. *Afr J Food Sci* 3: 340–346.

29. Giraffa G. (2002) Enterococci from foods. *FEMS Microbiol Rev* 26: 163–171.
30. Barbosa J, Ferreira V, Teixeira P (2009) Antibiotic susceptibility of enterococci isolated from traditional fermented meat products. *Food Microbiol* 26: 527–532.
31. Ribeiro T, Oliveira M, Fraqueza MJ, et al. (2011) Antibiotic resistance and virulence factors among enterococci isolated from chouriço, a traditional Portuguese dry fermented sausage. *J Food Prot* 74: 465–469.
32. Flores M, Corral S, Cano-García L, et al. (2015) Yeast strains as potential aroma enhancers in dry fermented sausages. *Int J Food Microbiol* 12: 16–24.
33. Felício MTS, Hogg T, Gibbs P, et al. (2007) Recurrent and sporadic *Listeria monocytogenes* contamination in *alheiras* represents considerable diversity, including virulence-attenuated isolates. *App Environ Microbiol* 73: 3887–3895.
34. Felício MTS, Ramalheira R, Ferreira V, et al. (2011) Thermal inactivation of *Listeria monocytogenes* from *alheiras*, traditional Portuguese sausage during cooking. *Food Control* 22: 1960–1964.
35. Castro A, Santos C, Meireles H, et al. (2016) Food handlers as potential sources of dissemination of virulent strains of *Staphylococcus aureus* in the community. *J Infect Public Health* 9: 153–160.
36. Friedman M, Henika PR, Levin CE, et al. (2007) Recipes for antimicrobial wine marinades against *Bacillus cereus*, *Escherichia coli* O157:H7, *Listeria monocytogenes*, and *Salmonella enterica*. *J Food Sci* 72: M207–M213.
37. Jahan M, Krause DO, Holley RA (2013) Antimicrobial resistance of *Enterococcus* species from meat and fermented meat products isolated by a PCR-based rapid screening method. *Int J Food Microbiol* 163: 89–95.
38. Valenzuela AS, Ben-Omar N, Abriouel H, et al. (2009) Virulence factors, antibiotic resistance, and bacteriocins in enterococci from artisan foods of animal origin. *Food Control* 20: 381–385.
39. Fraqueza MJ (2015) Antibiotic resistance of lactic acid bacteria isolated from dry-fermented sausages. *Int J Food Microbiol* 212: 76–88.
40. Federici S, Ciarrocchi F, Campana R, et al. (2014) Identification and functional traits of lactic acid bacteria isolated from Ciauscolo salami produced in Central Italy. *Meat Sci* 98: 575–584.
41. Gevers D, Huys G, Swings J (2003) *In vitro* conjugal transfer of tetracycline resistance from *Lactobacillus* isolates to other Gram positive bacteria. *FEMS Microbiol Lett* 225: 125–130.
42. Marty E, Bodenmann C, Buchs J, et al. (2012) Prevalence of antibiotic resistance in coagulase-negative staphylococci from spontaneously fermented meat products and safety assessment for new starters. *Int J Food Microbiol* 159: 74–83.
43. Rebecchi A, Pisacane V, Callegari ML, et al. (2015) Ecology of antibiotic resistant coagulase-negative staphylococci isolated from the production chain of a typical Italian salami. *Food Control* 53: 14–22.
44. Pereira V, Lopes C, Castro A, et al. (2009) Characterization for enterotoxin production, virulence factors, and antibiotic susceptibility of *Staphylococcus aureus* isolates from various foods in Portugal. *Food Microbiol* 26: 278–282.
45. Miranda JM, Guarddona M, Vázquez BI, et al. (2008) Antimicrobial resistance in *Enterobacteriaceae* strains isolated from organic chicken, conventional chicken and conventional turkey meat: A comparative survey. *Food Control* 19: 412–416.
46. Todorov SD, Stojanovski S, Iliev I, et al. (2017) Technology and safety assessment for lactic acid bacteria isolated from traditional Bulgarian fermented meat product "lukanka." *Braz J Microbiol* 48: 576–586.

47. Jimoh SO, Shittu AA, Morhason-Bello I (2013) Occurrence of virulence factor and extended spectrum beta lactamase in *Enterobacteriaceae* associated with ready-to-eat-fruits. *Int J Biol Biol Sci* 2: 083–087.
48. European Food Safety Authority (2011) Scientific opinion on risk based control of biogenic amine formation in fermented foods. Panel on Biological Hazards (BIOHAZ). *EFSA J* 9: 2393.
49. Latorre-Moratalla ML, Bover-Cid S, Talon R., et al. (2010) Distribution of aminogenic activity among potential autochthonous starter cultures. *J Food Prot* 73: 524–525.
50. Durlu-Özkaya F, Ayhan K, Vural N (2001) Biogenic amines produced by *Enterobacteriaceae* isolated from meat products. *Meat Sci* 58: 163–166.
51. Martín B, Garriga M, Hugas M, et al. (2006) Molecular, technological and safety characterization of Gram-positive catalase-positive cocci from slightly fermented sausages. *Int J Food Microbiol* 107: 148–158.
52. Latorre-Moratalla ML, Bover-Cid S, Veciana-Nogués MT, et al. (2012) Control of biogenic amines in fermented sausages: role of starter cultures. *Front Microbiol* 3: article 169.

AIMS Press