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Table of Contents: Volume 10 Number 13 7 April, 2016

ARTICLES

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Full Length Research Paper

Optimization of cellulase production for *Bacillus* sp. and *Pseudomonas* sp. soil isolates

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This investigation deals with optimizing the cellulase producing bacterial isolates for better enzyme yield. Cellulose degrading bacteria were isolated from garden soil (Aurangabad, MS, India). Amongst 40 different bacterial isolates, two of the bacterial isolates, 2b and 38b, were processed for preliminary identification with morphological, cultural and biochemical characterization. Based on these studies, strain 2b was qualified to belong to *Pseudomonas* sp. and strain 38b was qualified to belong to *Bacillus* sp. Optimization of the fermentation medium for maximum cellulase production was carried out for both strains of 2b and 38b. The culture conditions such as: pH, temperature, substrate concentration and incubation time were optimized. The optimum conditions found for cellulase production were 30°C at pH 5. Studies on partially purified cellulase, a high cellulolytic activity was observed in *Pseudomonas* sp. The Km 22.11 and 14.36 were obtained for cellulase from 2b and 38b, respectively. The Vmax was found to be 1 and 1.121 mmol (min mg)$^{-1}$ for cellulase from 2b and 38b , respectively.

Key words: *Bacillus* sp., cellulase production, pH, *Pseudomonas* sp.

INTRODUCTION

Cellulose is the primary product of photosynthesis in terrestrial environments and the most abundant renewable bioresource produced in the biosphere (Jarvis, 2002; Zhang and Lynd, 2004). Plants are the most abundant source of cellulose and are found as microfibrils (“2-20 nm” in diameter and “100-40,000 nm” in length). Cellulase is one of the enzymes produced mainly by fungi, bacteria and protozoans that catalyze cellulolysis (the decomposition of cellulose and of some related polysaccharides). In spite of the high growth rates of bacteria, they do not have enough cellulase production as with the fungi. Though several microorganisms are now known as cellulase producers, a relatively few numbers of fungi and bacteria are known to produce the enzyme in high levels. So far, most of the studies have been focused on the cellulase producing fungi (Callow et al., 2016; Sharma et al., 2015; Saini et al., 2015; Lan et al., 2013), bacteria (Shanmugapriya et al., 2012; Assareh

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et al., 2012; Rastogi et al., 2010) and actinobacteria (Cirigliano et al., 2013; Sarita et al., 2013). However, the application of bacteria in producing cellulase is not widely used (Bhat, 2000). The cellulolytic property of some bacterial genera such as *Cellulomonas, Cellvibrio, Pseudomonas* (Nakamura et al., 1982), *Bacillus* and *Micrococcus* has been reported (Immanuel et al., 2006).

Enzyme production is closely controlled in micro-organisms and to improve its productivity, these controls can be ameliorated. Cellulase yields appear to depend upon a complex relationship involving a variety of factors such as inoculum size, pH value, temperature, presence of inducers, medium additives, aeration and growth time (Immanuel et al., 2006). Enormous amounts of agricultural, industrial and municipal cellulosic wastes are accumulating or used inefficiently due to their high cost towards utilization processes (Lee et al., 2008).

Therefore, this problem is arising as a topic of considerable economic interest to develop processes for the effective treatment and utilization of cellulosic wastes as inexpensive carbon sources. Cellulose containing wastes may be agricultural, urban, or industrial in origin, and in addition, sewage sludge might also be considered as a source of cellulose since its cellulosic content provides the carbon for methane production in the anaerobic digestion of the sludge. This study primarily focused on obtaining an efficient degradation of cellulose from garden soil bacteria and this was achieved by optimizing the physical conditions (pH, temperature, etc) and nutritional requirements (substrate concentration) with respect to time.

MATERIALS AND METHODS

Isolation and screening of bacteria

Isolation of microorganisms from garden soil

The cellulolytic bacteria were isolated from garden soil. The damp and wet garden soil was used for isolation of cellulose degrading bacteria. The CMC medium was used for the enrichment and isolation of microorganisms. About one gram soil was inoculated into total 10 ml CMC broth (w/v), incubation for enrichment was carried out for 15 days at room temperature. After 15 days of incubation, turbid culture was serially diluted (10 fold) in physiological saline (sterilized 0.85% NaCl). To obtain single colonies of cellulose degraders, serial dilutions were spread plate inoculated onto the CMC agar. At the same time, enriched suspension was directly streak inoculated onto separate CMC agar plate. These plates were then incubated at room temperature for the appearance of single colonies. To visualize the hydrolysis zone (cellulose production), the plates were flooded with an aqueous solution of 0.1% Congo red for 15 min and washed with 1 M NaCl (Apun et al., 2000).

To indicate the cellulolytic activity of the organisms, diameter of hydrolytic zone around the growing colony on CMC agar was measured. Isolate qualifying for cellulose production was then used for quantification of cellulose activity in liquid medium. The cellulase activity of each culture was measured by determining the amount of reducing sugars liberated by using a di-nitro salicylic acid (DNSA) colorimetric method (Miller, 1959). Bacterial isolates 2b and 38b exhibiting relatively high enzyme activity of other isolates were selected for optimization of cellulose production.

Bacterial identification

The bacterial isolates were presumptively identified by means of morphological examination and some biochemical characterizations. The parameters investigated included colonial morphology, Gram's reaction, endospore formation, catalase production, Voges Proskauer (VP) reaction, indole production, starch hydrolysis, citrate utilization and gelatine hydrolysis. The results were compared with Bergey's Manual of Determinative Bacteria (Buchanan et al., 1974).

Production of cellulase

The bacterial isolates 2b and 38b were used in the cellulase production and optimization studies. The CMC broth (50 ml) in 500 ml flask was inoculated and the cellulase production was carried out. The CMC broth used was a minor modified version of ATCC medium 2270. In brief, for 1 L medium $(NH_4)2SO_4$ 1.0 g, $MgSO_4$ x $7H_2O$ 1.0g, $CaCl_2$ x $2H_2O$ 1.0 g, $FeCl_3$ 0.2 g, K_2HPO_4 (filter) 1.0 g, Casitone 2.0 g, carboxymethyl cellulose 15.0 g (Agar 15.0 g), was digested in double distilled water, 900 ml. Upon autoclaving, the pH was adjusted by supplementing with required sterile KH_2PO_4 solution and sterile double distilled water to make up 1 L. After 6 days incubation at room temperature, the cellulase was recovered in cell free culture supernatant by centrifugation at 1000 rpm for 25 min. The crude enzyme was extracted with 0.025 M sodium citrate buffer of pH 5.6 at 4°C and was used further in enzyme kinetics study.

Enzyme assay

For the assay, crude enzyme (2 ml) was mixed with 1% (w/v) cellulose in 0.05 mM sodium citrate buffer Ph 5. Reaction volume was made up to 5 ml by adding deionised water. The reaction mix was incubated at 30°C for 2 h. The reducing sugar product was determined by the DNSA method. Amount of released reducing sugar was estimated by plotting optical density on standard plot obtained by performing DNSA reaction on various glucose concentrations.

Process optimization for maximum cellulase production

pH

The growth medium containing the optimum concentration of substrate and carbon source was used. The pH of the broth was optimized by performing the reaction at different pH. Both 2b and 38b isolates were allowed to grow in media of different pH: 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5 and 8.0. At the end of incubation period, a cell-free culture supernatant was used as the source of enzyme.

Temperature

Production medium at pH 5 was inoculated with overnight grown bacterial strain. The broth was incubated at different temperatures of 25, 30, 35 and 40°C for 24 h. At the end of incubation period, the cell-free culture supernatant was used as the source of enzyme.

Substrate

Production medium at pH 5 was inoculated with overnight grown bacterial strain. The substrate concentration: 0.5, 1.0, 1.5, 2.0 and

4.0 mg/ml were used in separate flasks and incubated at 30°C for 24 h. At the end of incubation period, the cell-free culture supernatant was used as the source of enzyme.

RESULTS

Screening of isolates for the enzyme activity

About 40 bacterial isolates exhibiting cellulose degradation were isolated. Results shown in Table 1 and Figure 1 denote qualitative ability of these isolates needed for cellulase production. It is clear from the table that isolate 2b and 38b were found to be promising towards cellulase production ability in comparison with other isolates from this study and hence optimization for the enzyme production was studied with these two isolates, only.

Identification of bacterial isolates

Once it was noticed that amongst 40 different bacteria producing cellulase, the 2b and 38b strains were promising and we sought to address their preliminary characterization. Studies were performed to understand the morphological, cultural and characteristics and the results are shown in Table 2. Based on morphological, cultural and biochemical characters, the isolate 2b and 38b were putatively identified as *Pseudomonas* sp. and *Bacillus* sp. respectively.

Determination of enzyme activity

Results shown in Figure 2 show that isolate 2b and 38b were found to be active for cellulolytic activity after 40 min of reaction incubation at room temperature and at pH 5. However, the optimum reaction rate was observed after 60 min of incubation. ~1 and 0.9 µm/min/mg enzyme specific activity was found in crude extracts for 2b and 38b, respectively. As optimum enzyme activity was noticed with 60 min incubation, it was considered as optimum time for the reactions in further experiments.

Optimization of pH for the production of cellulase

Both 2b and 38b isolates were found to produce maximum enzyme activity at 60 min, we then sought to identify optimum pH for the cellulase production. Towards this, the pH of the broth was optimized by using the reaction buffer of different pH. Both 2b and 38b isolates were allowed to grow in media of different pH: 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5 and 8.0. Observations depicted in Figure 3 indicate that crude cellulase from 2b and 38b was found to be more active at pH 5.0. The pH 5 of growth medium and reaction mix was used in our future experiments. Our results are in agreement with the finding of other workers (Chantawannakul et al., 2002; Abdel-Mawgoud et al., 2008) who also used *Bacillus subtilis* as the model organism.

Table 1. Ability to produce cellulase, identified with the help of hydrolysis zone.

S/N	Bacterial isolates	Cellulase activity
01	1a	+
02	1b	-/+
03	2a	+
04	2b	++++
05	4a	+
06	4b	+
07	6	-
08	7a	-
09	7b	-
10	9	-
11	11	-
12	12a	-
13	12b	-
14	13	-
15	14a	-
16	14b	-
17	14c	-
18	15	-
19	16	+/-
20	21	++
21	22a	-
22	22b	-
23	27	+
24	28	+++
25	29a	+
26	29b	+++
27	30a	+
28	30b	+
29	31	-
30	32	-
31	33	++
32	36a	-
33	36b	-
34	36c	-
35	38a	+
36	38b	++++
37	38c	+
38	39	+
39	40a	+
40	40b	+++

- Negative for cellulase production, +, ++ and ++++ qualitative activity.

Optimization of temperature for the cellulase production

In the above experiment, it was shown that cellulase

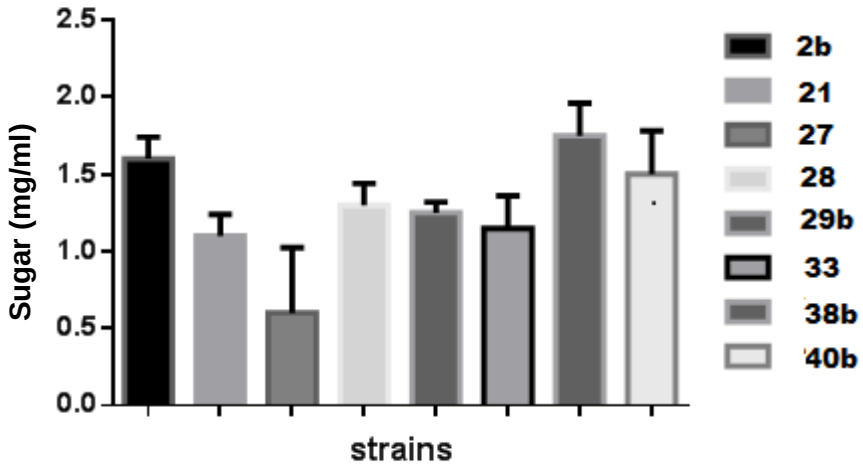

Figure 1. Screening of bacterial isolates for the production of cellulase. Total residual sugar was estimated from the growth medium of the bacterial. The cellulase activity was determined by DNSA method. Sugar concentration, 1.2 mg/ml was considered significant for the positive selection of the isolate.

Table 2. Biochemical characterization of cellulase producing bacteria.

S/N	Biochemical test	*Pseudomonas* sp.2b	*Bacillus* sp.38b
1	Grams nature	Gram negative rods	Gram positive rods
2	Motility	Motile	Motile
3	Indole production	Positive	Negative
4	Methyl Red	Negative	Positive
5	VogesProskauer	Positive	Positive
6	Citrate Utilization	Positive	Negative
7	Oxidase	Positive	
8	Endosopre formation	Negative	Positive, central non bulging spore
9	Catalase	Positive	Positive
10	Gelatin Hydrolysis	Positive	Positive
11	Starch Hydrolysis	ND	Positive
12	Urease	Positive	Negative
13	Nitrate Reduction	ND	ND
14	H_2S Production	Negative	Negative
15	Carbohydrate fermentation tests	-	-
a	Arabinose	Negative	Positive
b	Xylose	Negative	Positive
c	Maltose	Negative	Positive
d	Sucrose	Negative	Positive
e	Mannitol	ND	Positive
f	Glucose	Positive	Negative
g	Lactose	Negative	Negative

activity was found to be produced better within 60 min and at pH 5.0. The authors sought to address the optimum temperature for the production of cellulose, enzyme activity was recorded at different temperatures. Results shown in Figure 4 depicts tha both 2b and 38b strains produced maximum cellulase production at 30°C. The 30°C incubation temperature was thus used for the ES reactions in further experiments. The temperature was found to influence extracellular enzyme secretion, possibly by changing the physical properties of the cell

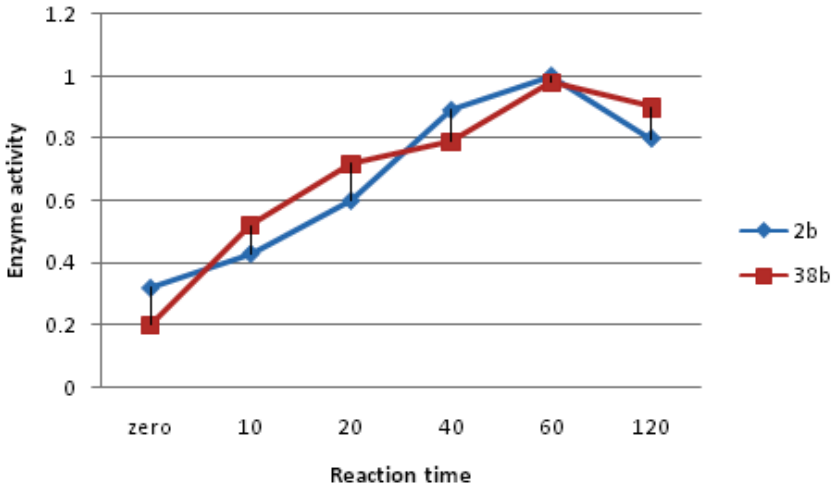

Figure 2. The enzyme specific activity of cellulase (µm/min/mg) vs. time. 2b and 38b were used for the cellulase production. The enzyme specific activity of isolated enzyme was determined at different incubation time. The optimum reaction rate was observed at 60 min incubation. Measurements were made in triplicate, and standard bars represent the standard deviation.

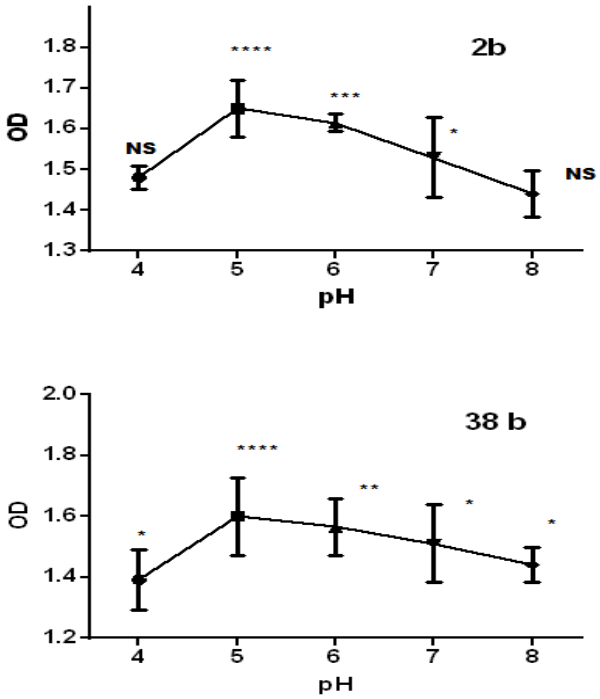

Figure 3. The production of cellulase at optimum pH. 2b and 38b were used for the cellulase production. The production of cellulase was determined at different pH. The pH of the broth was optimized by using the reaction buffer of different pH. Both 2b and 38b isolates were allowed to grow in media of different pH: 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5 and 8.0. Measurements were made in triplicate, and standard bars represent the standard deviation. The Student's two-tailed t-test was used to determine statistical significance of the differences between enzyme activities at different time interval. Error bars indicate SD (set as 100%; n = 3; ****P <0.0001, ***P <0.001, **P <0.01, *P < 0 .05, ns-not significant).

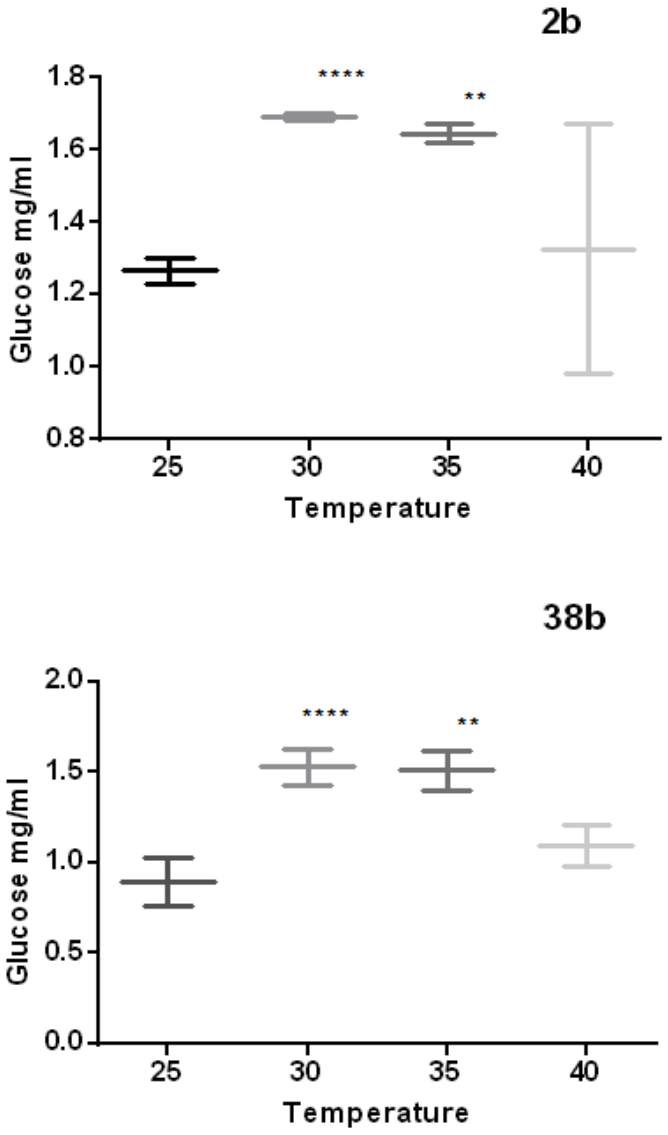

Figure 4. The optimization of temperature for the production of cellulase. 2b and 38b were used for the cellulase production. The enzyme production was recorded at different temperatures. Results depict that 2b and 38b strains produced maximum cellulase production at 30°C. The optimum reaction rate was observed at 30°C and pH 5. Measurements were made in triplicate, and standard bars represent the standard deviation. The Student's two-tailed t-test was used to determine statistical significance of the differences between enzyme activities at different time interval. Error bars indicate SD (set as 100%; n = 3; ****P <0.0001, ***P <0.001, **P <0.01, *P < 0 .05, ns-not significant).

membrane. This observation is in agreement with earlier report on *Bacillus subtilis* by Jansova et al. (1993).

Incubation time and the cellulase production

After optimizing reaction conditions for enzyme activity, the authors sought to grow strain 2b and 38b in growth medium and study the enzyme production with reference to incubation time. Crude cell free supernatant were collected every 24 h until 120 h incubation. It is evident from the Figure 5 that in case of strain 2b significant enzyme production was detected at the end of 24 h and slight increase was noticed at 48 h incubation. Further

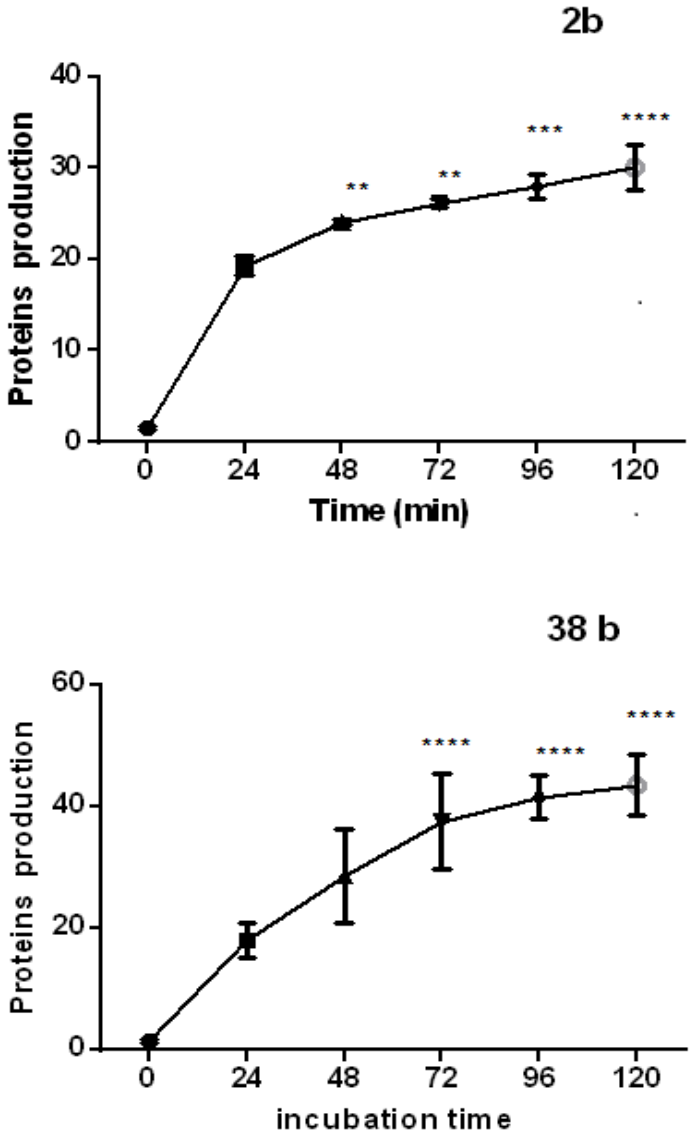

Figure 5. Cellulase production at various time durations. 2b and 38b were used for the cellulase production. The enzyme quantity was measured at various time point. Maximum protein production was observed at 120 min after incubation. Measurements were made in triplicate, and standard bars represent the standard deviation. The Student's two-tailed t-test was used to determine statistical significance of the differences between enzyme activities at different time interval. Error bars indicate SD (set as 100%; n = 3; ****P <0.0001, ***P <0.001, **P <0.01, *P < 0 .05, ns-not significant).

incubation did neither increased the enzyme production nor was it affected (Figure 5). On the other hand, strain 38b produced enzyme at 24 h time point and continued to exhibit elevated enzyme activity until last time point, 120 h. However, increased enzyme production by 38b strain beyond 48 h was not significant enough (Figure 5). The optimum reaction rate was observed after 24 h incubation.

Cellulase kinetic studies

Effect of substrate concentration on cellulase activity

The crude cellulase obtained from 2b and 38b strains was found to be active at 30°C and at pH 5. The rate of reaction was found to increase with increase in the substrate concentration, shown in Figure 6. The maximum

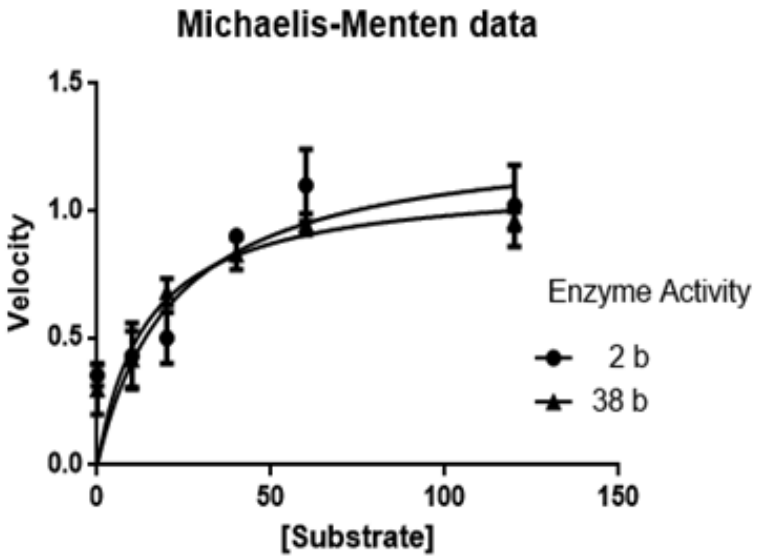

Figure 6. Enzyme kinetics of cellulase isolated from 2b and 38b. Cellulase from 2b and 38b were used for the Michaelis-Menten kinetics. The enzyme specific activity of isolated enzyme was determined at various concentrations of substrates. The Km was determined by using the GraphPad Prism 6.01. The Km 22.11 and 14.36 was obtained for cellulase from 2b and 38b, respectively. The Vmax was found to be 1.3 and 1.121 mmol(min mg)$^{-1}$ for cellulase from 2b and 38b, respectively. Measurements were made in triplicate, and standard bars represent the standard deviation.

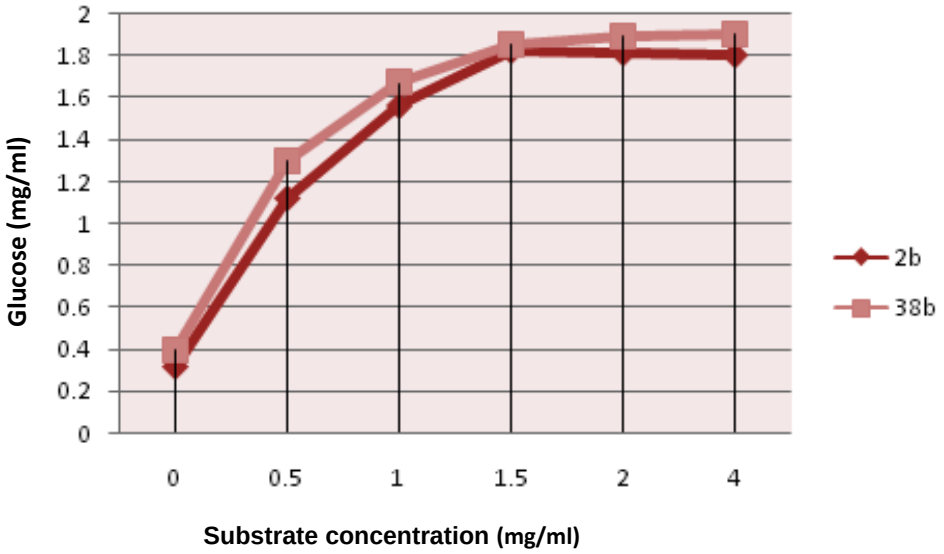

Figure 7. Optimization of substrate concentration for the cellulase. 2b and 38b were used for the cellulase production. The enzyme specific activity of isolated enzyme was determined at various concentrations of substrates. Measurements were made in triplicate, and standard bars represent the standard deviation.

activity at 30°C, pH 5, with 60 min was found with 1.5 mg substrate concentration shown in Figure 7. The Km was estimated with the use of GraphPad Prism 6.01. The Km for strains 2b and 38b was 22.11 and 14.36, respectively. The Vmax was found to be 1.3 and 1.121 mmol/min/mg for the cellulase enzyme from 2b and 38b, respectively.

Maximum enzyme activity at 48 h was found with 1.5 mg substrate concentration in cell free supernatant of 2b and 38b, respectively.

DISCUSSION

Treatment of cellulose by cellulolytic enzymes for practical purposes has attracted the continuing interest of biotechnologists. In this study, the production of cellulase from bacterial isolates was optimized. The production of cellulase from *Pseudomonas* sp. 2b and *Bacillus* sp. 38b strains was achieved.

Lignocellulosic biomass, (plant biomass), is a great potential resource for the production of biofuels because it is largely abundant, inexpensive and production of such resources is environmentally sound. Agricultural residues are a great source of lignocellulosic biomass which is renewable, chiefly unexploited, and inexpensive. Such resources include: leaves, stems and stalks from sources such as corn fibre, corn stover, sugarcane bagasse, rice hulls, woody crops and forest residues. Also, there are multiple sources of lignocellulosic waste from industrial and agricultural processes, e.g., citrus peel waste, sawdust, paper pulp, industrial waste, municipal solid waste and paper mill sludge. Both fungi and bacteria have been exploited for their abilities to produce a wide variety of cellulases and hemicellulases (Zhang and Lynd, 2004; Bhat, 2000; Nakamura et al., 1982; Sethi et al., 2013). Most emphasis has been laid on the use of fungi because of their capability to produce copious amounts of cellulases and hemicellulases which are secreted to the medium for easy extraction and purification.

Secondly, bacterial glycoside hydrolases are often more complex and expressed as multi-enzyme complex providing increased function and synergy. Most importantly, bacteria inhabit a wide variety of environmental and industrial niches, which produce cellulolytic strains that are extremely resistant to environmental stresses (Nakamura et al., 1982; Immanuel et al., 2006).

Further studies were in progress in the purification and application of cellulase in different commercial fields. The purified cellulase can be used for various purposes in detergent industries, food industries and pharmaceutical industries. The high activity and stability of cellulase enzymes between neutral to alkaline pH and high temperature will be of use in various industrial and biotechnological applications.

These results are close to those of Bakare et al. (2005) who found that the cellulase enzyme produced by *Pseudomonas fluorescence* was activated from 30 to 35°C showing the optimum temperature at 35°C. Ray et al. (2007) reported that minimum cellulase yield was observed when fermentation was carried out at 45°C, while maximum yield was obtained at 40°C by *B. subtilis* and *Bacillus circulans*. Immanuel et al. (2006) also recorded maximum endoglucanase activity in *Cellulomonas, Bacillus* and *Micrococcus* sp. at 40°C and neutral pH. In this context, 2b and 38b growth was found at 30°C. The growth of bacterial isolates at room temperature minimizes the expenditure on temperature control at the scale up of the process. This is an advantage on previously reported strains. The bacterial strains 2b and 38b was optimized for the production of cellulase at 30°C. This study will be a milestone in the production of the cellulase from bacterial isolates at room temperature.

Conflict of Interests

The authors have not declared any conflict of interests.

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Full Length Research Paper

Antibiotic resistance of *Enterococci* isolated from raw camel milk in the South West of Algeria

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Little information is available on the diversity and distribution of resistance and virulence factors in Enterococci isolated from camel milk. In this study, 33 samples of camel's milk collected from the south west region of Algeria were analyzed for the presence of *Enterococcus* spp. Twenty three (23) enterococcal isolates were recovered. These strains were identified by the API 20 STREP and the sodium dodecyl sulphate-polyacramide gel electrophoresis (SDS-PAGE) of whole cell protein at the species level: *Enterococcus faecalis* (n = 11), *Enterococcus faecium* (n = 8), *Enterococcus avium* (n = 2), *Lactococcus lactis* ssp *lactis* (n = 1) and *Streptococcus uberis* (n = 1). Fifteen (15) of the 23 isolates exhibited resistance to at least one of the tested antibiotics and six (6) of these 23 isolates were resistant to two antibiotics. None of the isolates were resistant to penicillin, ampicillin, or gentamicin. Resistance to vancomycin (VAN) was found in three (3) isolates which represent (13%), two *E. faecalis*, and one *E. faecium*. Six (26%) of *Enterococci* isolates were resistant to one of these antibiotics: erythromycin (ERI), tetracycline (TET) and rifampin (RIF). In conclusion, this is the first study to underline the importance of camel milk as a reservoir of *Enterococcus* spp. carrying resistance to vancomycin.

Key words: Camel milk, *Enterococcus*, sodium dodecyl sulphate-polyacramide gel electrophoresis (SDS-PAGE), antibiotic resistance.

INTRODUCTION

Enterococci are important members of gut communities in many animals and opportunistic pathogens that cause millions of infections annually. They are most frequently used as fecal indicator bacteria, or general indicators of fecal contamination, but they are also used as surrogates for pathogens and/or health effects in risk assessment and other modelling applications. These bacteria are widely distributed in a variety of environmental habitats, even when there is little or no input from human and/or animal fecal sources (Byappanahalli et al., 2012). In

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addition, they are considered as lactic acid bacteria found in traditional fermented foods and in the dairy products (Sukontasing et al., 2007).

The prevalence of enterococci in dairy products is a result of unhygienic conditions during the production and processing of milk. *Enterococci* may enter the milk either directly from human or animal feces or indirectly from contaminated water sources, exterior of the animal and/or from the milking equipment and bulk storage tank. Different species of enterococci are found in dairy products, but *Enterococcus faecalis* and *Enterococcus faecium* remain the species of greatest importance (Giraffa, 2003).

Data on the microbial diversity of raw camel milk are generally scarce compared with bovine milk. The microflora of raw and fermented camel milk products has been reported as a mix of different species of typical dairy bacteria (Christoph et al., 2012). This microflora needs to be further investigated. The environmental conditions where the milk is produced by camels and its physicochemical properties determine the group of microorganisms that can survive in such conditions. In this case, these conditions (temperature, pH, concentration of salt...) are ideal for the growth and proliferation of enterococci. The objectives of this study were to identify the species and describe the antimicrobial resistance features of *Enterococci* isolated from camels' milk.

MATERIALS AND METHODS

Sample collection

A total of 33 samples of camel's milk were collected from free range camel herd (*Camelus dromedarius*), in good health, living in the South West of Algeria (Bechar area). The milk was collected during the period of February, March and April, 2014 in sterile bottles, transported to the laboratory in an icebox and stored at +4 to +6°C before analysis. This work was performed in the biological laboratories, Department of Biology at the University of Bechar, Algeria.

Physiological and biochemical characterization of *Enterococcus* strains

Enterococcal isolates were obtained from camel's milk. Growth characteristics were tested in de Man-Rogosa-Sharpe agar (MRS), Citrate Azide agar (CA) and Citrate Azide Tween Carbonate medium (CATC) (Domig, 2003). These isolates were first phenotypically described by using conventional growth and physiological tests, according to Devriese et al. (2006). All cultures were examined for ability to grow on potassium tellurite 0.04%, for hydrolysis of esculin and for gelatine liquefaction. Production of hemolysis was determined by plating actively growing cells of the strains onto Columbia blood agar (Oxoid) supplemented with 5% (v/v) human blood. Plates were incubated at 37°C in an anaerobic atmosphere. Results were recorded at 24 and 72 h. A clear zone of β-hemolysis on blood agar plates was considered as positive result.

The type strain for *E. faecalis* ATCC 29212 was obtained from the American Type Culture Collection. Stock cultures were maintained on MRS broth supplemented with 30% glycerol and stored at -20°C. After that, all isolated strains were tested with API 20 STREP galleries according to the manufacturer's instructions (BioMérieux), and identified using the analytical profile index. Physiological and biochemical characteristics were coded as 0 for negative and 1 for positive and analyzed by the software package BioNumerics version 7.5 (Applied Maths, Kortrijk, Belgium). Agglomerative clustering was performed by the unweighted pair group method with arithmetic mean (UPGMA).

Analysis of whole-cell protein profiles by SDS-PAGE

Preparation of samples and analysis of whole-cell protein profiles by conventional one-dimensional sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) were performed as described by Merquior et al. (1994), with slight modification where the strains used for protein extraction were first plated onto CATC medium, then they were grown on brain heart infusion broth instead of Columbia blood agar. Coefficients of similarity or dice indices between isolates and the Enterococcal reference strain were determined for each isolate by using the BioNumerics version 7.5 software package (Applied Maths, Kortrijk, Belgium), and a dendrogram was constructed from the similarity matrix by the unweighted pair group method with arithmetic mean (UPGMA). The whole-cell protein extract of *E. faecalis* ATCC 29212 was used as reference profile.

Antibiotic susceptibility test

All isolates were tested for their antibiotic susceptibility by a disc diffusion method on Mueller-Hinton agar. Seven antibiotics were used: penicillin 10 U (PEN), ampicillin 10 µg (AMP), vancomycin 30 µg (VAN), erythromycin 15 µg (ERI), tetracycline 30 µg (TET), rifampin 5 µg (RIF) and gentamicin 120 µg (GEN). The diameter of inhibition zones were measured after incubation for 24 h at 35°C. Sensitivity and resistance were evaluated according to the Clinical and Laboratory Standards Institute guidelines (CLSI, 2014). Antibiotic resistance data expressed in millimeters (mm) of inhibition zone were first converted to categories (S for susceptible, I for intermediate, and R for resistant), then a dendrogram was constructed from the similarity matrix by the unweighted pair group method with arithmetic mean (UPGMA) using the BioNumerics version 7.5 software package (Applied Maths, Kortrijk, Belgium).

RESULTS

Isolation of *Enterococcus* strains

A total of 23 isolates of Enterococcal strains was isolated from camel's milk. As CATC medium is selective for enterococci, all Gram-positive, catalase-negative cocci isolated from this medium were presumptively identified as *Enterococcus* spp. The presumptive identification showed that all isolates were morphologically homogeneous, they were spherical or ovoid cells occurring in pairs or short chains, non-motile, and they were gram positive catalase negative.

Physiological and biochemical identification

All isolated strains showed the same physiological characteristics, they grew in MRS broth containing 6.5%

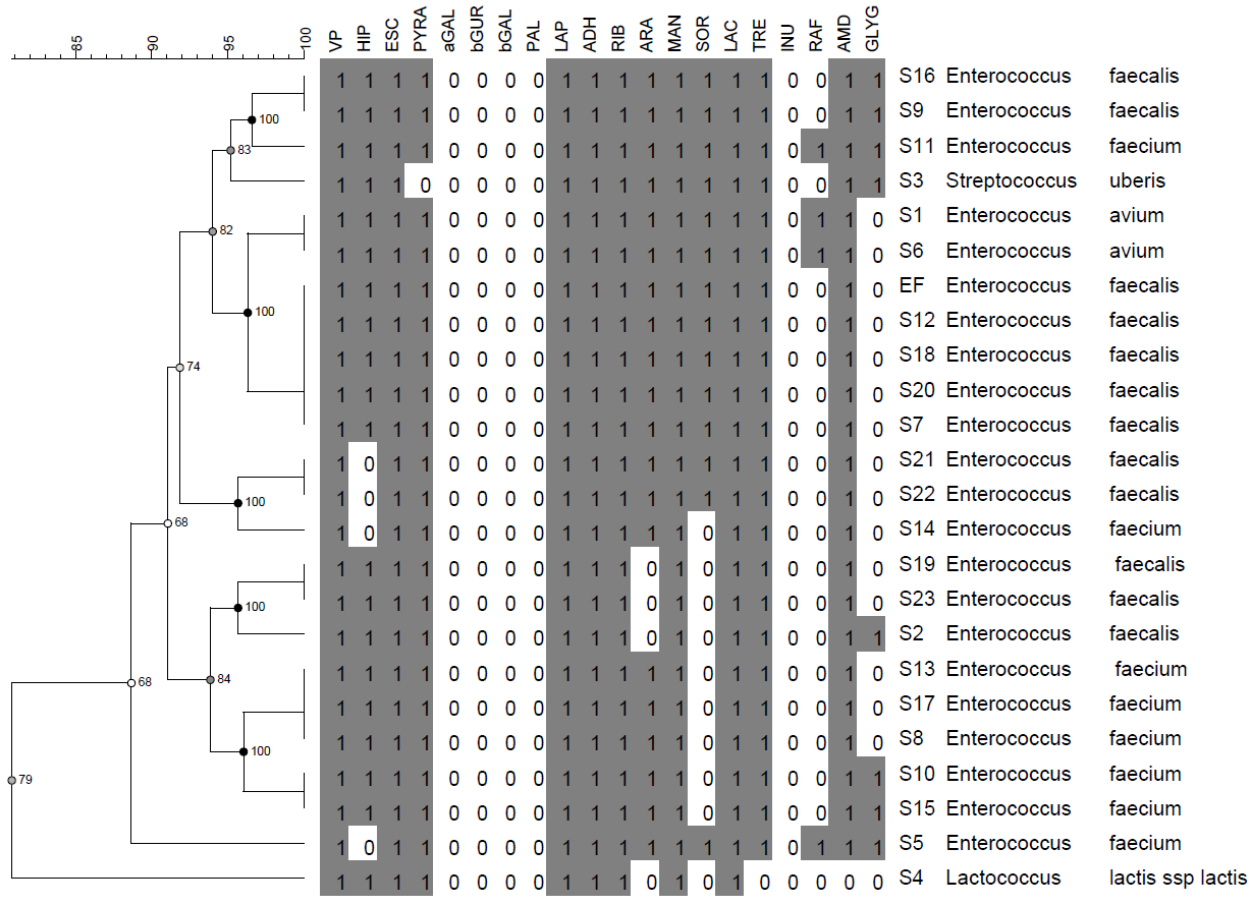

Figure 1. Results of the API 20 STREP tests represented as a clusters of biochemical profiles of the camel's milk enterococci isolates. (EF indicate reference strain).

NaCl, at pH 9.6 which is in accordance with the genus *Enterococcus*, and they grew at 10 and 45°C and resists 30 min at 63°C. They are positive for hydrolysis of esculin and negative for hydrolysis of gelatin and they do not show any tolerance for potassium tellurite. For hemolysis on blood agar they showed negative results.

API 20 strep system identification

All of the isolates were Vogues-Proskauer (VP), hippurate (HIP) (except for isolates: S5, S14, S21, and S22), esculin (ESC), pyrrolidonylarylamidase (PYRA) (except for isolate S3), leucine arylamidase (LAP), and arginine dihydrolase (ADH) positives, but negatives for alkaline phosphatase (PAL), α-galactosidase (α-GAL), β-glucuronidase (β-GUR), and β-galactosidase (β-GAL). With the exception of S4 which was unable to use trehalose and starch, all isolates were able to produce acid from ribose, mannitol, lactose, trehalose and starch by fermentation, but they were unable to produce acid from inulin. All the other tests were strain-dependent. These results were coded as 0 for negative and 1 for positive and analysed by the software package BioNumerics version 7.5 (Applied Maths, Kortrijk, Belgium), and clusters for species identification were depicted taking into account the clustering pattern of reference strain (Figure 1).

Whole-cell protein profiles identification

Whole-cell protein profiles of the isolates were compared with a type strain profile. Figure 2 shows a dendrogram that was obtained after UPGMA linkage cluster analysis of all the isolates and the type strains of *Enterococcus faecalis* ATCC 29212. Numerical analysis of the electrophoretic whole-cell protein profiles of the 23 camel's milk isolates and reference strain (*E. faecalis* ATCC 29212) by the determination of the dice correlation coefficient and UPGMA clustering, revealed that at the 65% similarity (S) level, the 23 isolates formed three distinct clusters as shown in the dendrogram (Figure 2). Cluster 1 with (71% r-value) grouped 8 isolates, five

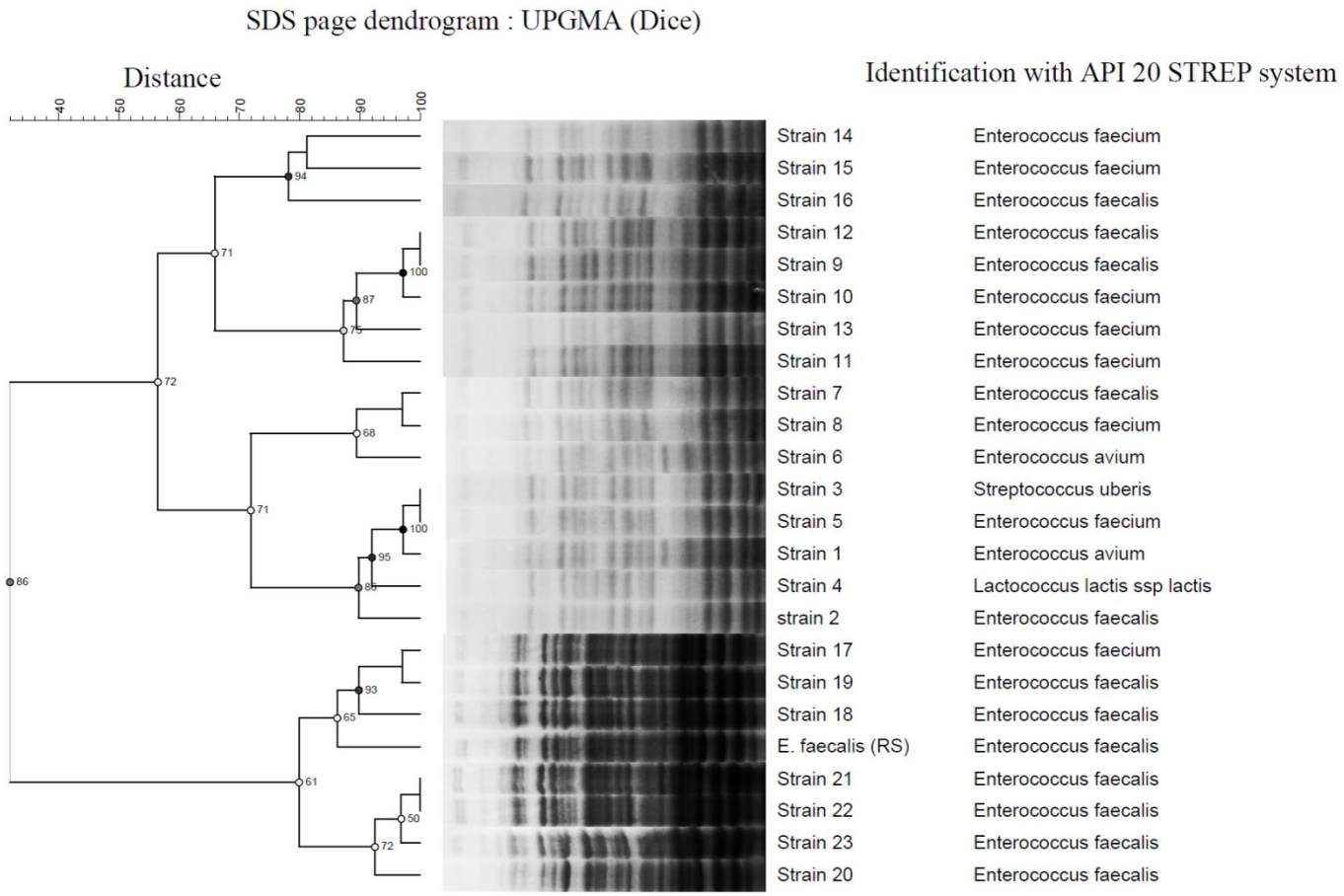

Figure 2. Electrophoretic banding patterns of whole cell protein of *Enterococcus* isolates; the mean correlation coefficient (r), represented as a dendrogram, and calculated by the unweighted average pair grouping method for some of the camel milk isolates compared with the reference strain. (RS indicates reference strain).

(S10, S11, S13, S14, and S15) were identified by the API 20 STREP system as *Enterococcus faecium*, and three (S9, S12, S16) were identified by the same system as *E. faecalis*. Cluster 2 with (71% r-value) grouped 8 isolates, two (S2, S7) were identified as *E. faecalis*, two (S5, S8) were identified as *E. faecium*, another two isolates (S1, S6) were identified as *E. avium*, one isolate (S3) was identified as *Streptococcus uberis*, and the last one (S4) was identified as *Lactococcus lactis ssp lactis*. Cluster 3 with 61% r-value also grouped 7 isolates with the reference strain, six isolates (S18, S19, S20, S21, S22, and S23) were identified as *E. faecalis*, and one isolate (S17) was identified as *E. faecium*.

Antibiotic susceptibility

Analysis of the antibiotic susceptibility of the isolates revealed that 15 of the 23 isolates exhibited resistance to at least one of tested antibiotics and 06 of these 23 isolates were resistant to two antibiotics. None of the isolates were resistant to penicillin, ampicillin, or gentamicin. Resistance to vancomycin was found in three isolates, two (S2, S19) were identified as *E faecalis*, and the other one (S5) was identified as *E. faecium*. Six isolates were resistant to erythromycin, two were identified as *E. faecalis* (S21, S23), two were identified as *E. faecium* (S10, S17), one was identified as *Streptococcus uberis*, and the last one isolate was identified as *L. lactis ssp lactis*. Six isolates were resistant to rifampin, three were identified as *E. faecalis* (S2, S18, S22), and the remaining three were identified as *E. faecium* (S11, S13, S17). Six isolates were found to be resistant to tetracycline, four were identified as *E. faecalis* (S16, S18, S19, and S22), one as *E. faecium* (S10), and the other one as *E. avium* (S1). It was found that only 34.78% of the isolates were susceptible to all tested antibiotics. The susceptibility results and patterns of all isolates tested are shown in (Table 1 and Figure 3).

DISCUSSION

Camel milk is a valuable product for the nomads in the

Table 1. Antibiotic susceptibility of enterococcal strains isolated from Camels' milk (n=23).

Antibiotics	Resistant (%)	Intermediate (%)	Sensitive
Ampicillin (10 µg)	0 (0)	4 (17)	19 (83)
Erythromycin (15 µg)	6 (26)	16 (70)	1 (4)
Gentamicin (120 µg)	0 (0)	0 (0)	23 (100)
Penicillin (10 U)	0 (0)	6 (26)	17 (74)
Rifampin (5 µg)	6 (26)	9 (39)	8 (35)
Tetracycline (30 µg)	6 (26)	3 (13)	14 (61)
Vancomycin (30 µg)	3 (13)	9 (39)	11 (48)

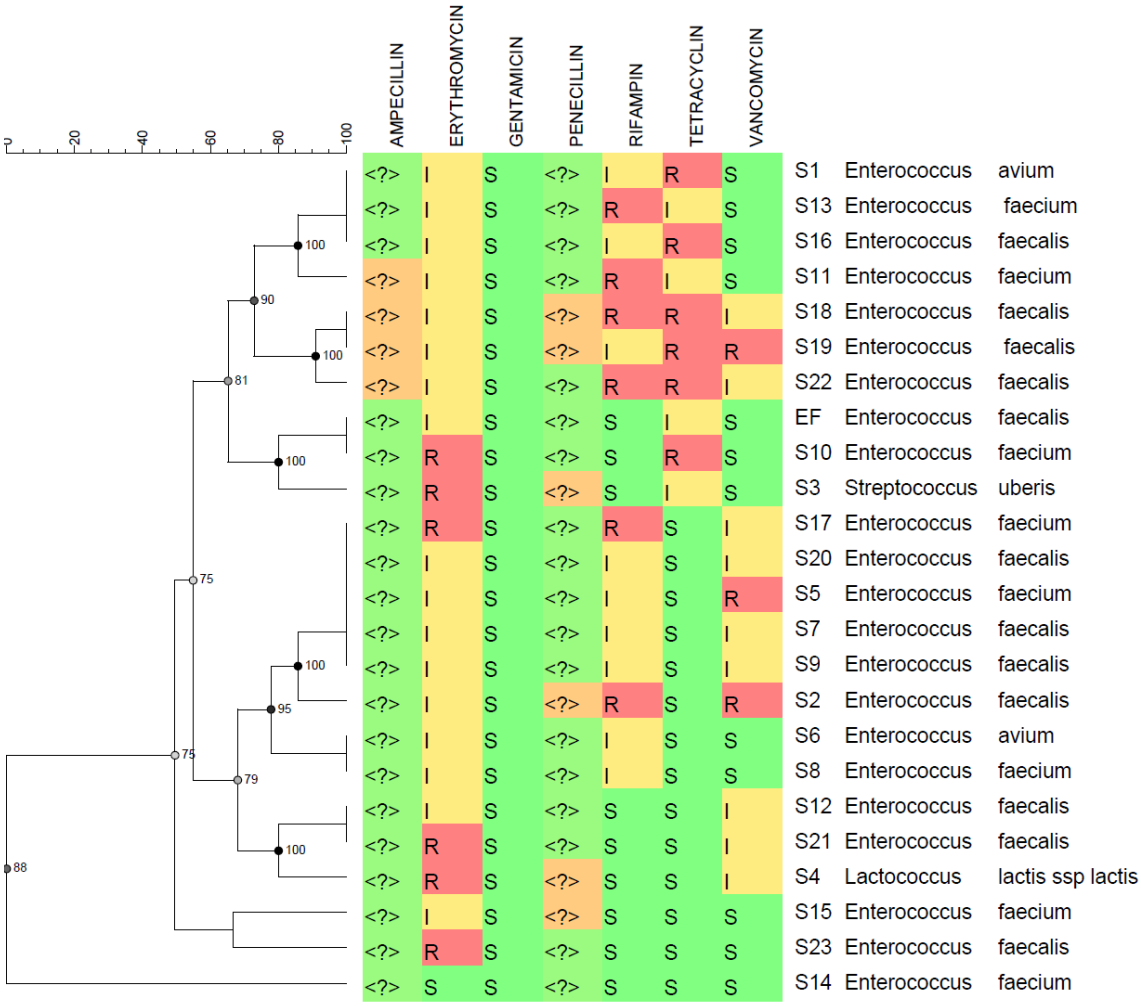

Figure 3. Antibiotic resistance profiling; *Enterococci* isolates are typically clustered based on their resistance categories using a categorical coefficient, which treats different values as different states. The colors in the comparison window correspond to the color of each antibiotic category (susceptible, intermediate or resistant). (EF indicate reference strain).

hot regions and arid countries, consumed as fresh and soured milk, this product has long been ignored and under-estimated, and it has not had its share of chance in the scientific research, when comparing it with the cow's milk that has been widely studied. The research done so far on the camel milk do not cover all aspects, studies carried out between 1997 and 2009 have been mainly concentrated on the composition, characteristics and

functionality of the camel milk (Al haj and Al Kanhal, 2010). Nevertheless, the data on the microbial diversity of camel milk are insufficient. For this purpose, an attempt was made in the current study to identify the enterococci present in camel's milk at species level and to investigate some potential pathogenic factors of these bacteria, such as hemolysis on human blood, and antibiotic resistance. The choice of enterococci was established based on their capacity to withstand harsh conditions such as drying, heat stress, and UV irradiation prevailing in the regions from where camel's milk samples were taken.

Analysis of results from physiological and biochemical tests was performed to determine characteristics that are usually considered as typical for the genus *Enterococcus*, such as growth at 45°C, 10°C, pH 9.6 and with 6.5% NaCl, and to allow a preliminary characterization of the isolates. Our isolates have given the same results of physiological tests which allow to classify them in the genus *Enterococcus* without exception, while the results of biochemical tests obtained from the API 20 STREP system have shown some differentiation between isolates, in which 11 (48%) species were identified as *E. faecalis*, 8 (35%) species as *E. faecium*, 2 (9%) species as *E. avium*, 1 (4%) species as *L. lactis ssp lactis*, and 1 (4%) species as *S. uberis*.

A number of studies on the API 20 Strep method showed that the majority of *E. faecalis*, *E. faecium*, *E. avium* and *E. durans* strains isolated of a clinical origin are correctly identified (Winston et al., 2004). However, because this system was developed prior to the recent taxonomy changes, some identifications may be in error, especially for species other than *E. faecalis* and for "*Enterococcus*-like" strains (Maria et al., 2002).

Clearly, a reliable identification of enterococci to the species and strain level by physiological and biochemical tests often appears difficult. Besides being very time consuming, this type of work yielded results that, in terms of a taxonomic identification, did not always match the results obtained by other methods. However, given the variability in the biochemical and phenotypic traits of enterococci, molecular based methods are essential for reliable and fast identification. SDS-PAGE analysis of whole-cell protein patterns is useful for clearly discriminating a multitude of species of lactic acid bacteria (Descheemaeker et al., 2000). It is equally possible to differentiate and identify *Enterococcus* species (Merquior et al., 1994). To clarify the identification of our isolates, electrophoretic analysis of the whole-cell protein profiles was performed. The profiles generated are shown in Figure 2. The results obtained show some discrepancies between results obtained by conventional phenotypic, API 20 STREP and SDS page profiling. Merquior et al. (1994) used and evaluated SDS-PAGE to identify reference, human, animal and environmental strains of *Enterococcus* species. They reported that each *Enterococcus* species had a unique and distinguishable profile. However, the limit of SDS-PAGE of the whole-cell protein profiles is that it requires several type of strains to clearly identify all isolates, our study is limited to one type strain *E. faecalis* ATCC 29212. Despite this it was found that 58% of isolates identified by API 20 STREP as *E. faecalis* were confirmed by whole-cell protein profiling that they belong to this species. The remaining 42% of isolates requires the use of other reference strains. Application of the whole-cell protein profiles analysis for enterococcal characterization requires standardization of reference banding patterns. In addition, a data bank of reference protein profiles could be constructed with which the protein profile of any unknown isolate could be compared. Whole-cell protein electrophoresis has widely been documented in numerous taxonomic and identification studies to be a reference method for species delineation because a high degree of similarity in whole-cell protein content is a reflection of a high degree of DNA homology, and therefore species identity (Vandamme et al., 1996).

The precise differentiation of enterococcal species has taken on additional importance because of the acquisition of resistance traits among strains, especially resistance to glycopeptides. To the best of our knowledge, this study provides the first detailed analysis about the ecology of antibiotic resistance and virulence in a variety of enterococci isolated from fresh raw camel milk in North Africa. The antibiotic susceptibility testing was performed according to standard disc diffusion method (Kirby–Bauer disc diffusion method) recommended by the Clinical and Laboratory Standards Institute (CLSI, 2014). Because of the limitation of techniques used to evaluate the antibiotic susceptibility, some studies have been conducted by Lee and Chung (2015), Edelmann et al. (2007) and Dickert et al. (1981) to determine the most appropriate method for antibiotic susceptibility testing, and they concluded that disk diffusion is still a valid technique and gives results that are closely similar to other techniques. It is important to develop an easy-to perform methodology that can be routinely used in the laboratory, but careful consideration regarding not only accuracy, but also cost and labor intensiveness is required.

Enterococci are known to acquire antibiotic resistance to most antibiotics used in clinical practice with relative ease and capable of spreading those resistance genes to other species (Kaçmaz and Aksoy, 2005). The occurrence of antibiotic resistance among dairy isolates seems to vary somewhat between studies, and is often strain- and region-dependent (Čanžek et al., 2005), or may differ according to the isolation method (Klein, 2003).

Our results of antibiotic susceptibility are summarized in Table 1 and Figure 3. They showed that 26% of enterococci isolates were resistant to one of these antibiotics erythromycin (ERI), tetracycline (TET), and rifampin (RIF). Teuber et al. (1999) found 64, 45 and 32% of resistance to chloramphenicol, tetracycline and

erythromycin, respectively, and they concluded that these antibiotics are a major concern for dairy *E. faecalis* isolates. Resistance to erythromycin as a representative of the macrolide antibiotics is a matter of concern. Although de Fatima Silva Lopes et al. (2005) have determined that a high percentage of *E. faecalis* strains (74%) were intermediary or resistant to erythromycin. In Poland, Wioleta et al. (2012) reported a similar prevalence of resistance to tetracycline (28.3% of isolated strains) to our study.

The most widespread resistance among dairy enterococci was tetracycline which was detected in 30.8% of the strains, this may be attributed to the widespread use of these antibiotics in veterinary practices (Pieniz et al., 2015). Huys et al. (2004) also showed that a significant proportion of tetracycline isolates exhibited co-resistance to erythromicin and/or chloramphenicol, suggesting that the selection of tetracycline genotypes may provide a suitable molecular basis for the further selection of multiple resistances. However, it should be noted that resistance to tetracycline has little clinical importance as it is not a drug of choice for the treatment of enterococcal infection. A major concern is the emergence of vancomycin resistant *Enterococci* (VRE). Vancomycin is considered as the last resort antibiotic to treat serious infections due to resistant Gram-positive bacteria, and given exclusively in a clinical environment, when all others fail. (Naoual et al., 2010).

Several studies showed the occurrence of vancomycin-resistant enterococci in food of animal origin, mainly in *E. faecalis* and *E. faecium* species, although the isolation frequency seems to be lower than in clinical samples (Klein, 2003). In our case three (3) vancomycin resistant enterococci (VRE) were found which represent 13% of all isolated strains, two (S2, S19) were identified as *E. faecalis*, and one (S5) was identified as *E. faecium*. According to Morandi et al. (2006), testing antibiotic susceptibility to vancomycin by disc diffusion method provide similar results as growth in MRS broth containing vancomycin.

Vancomycin resistance within dairy enterococci remains controversial, though several papers indicate very low or no presence of *vanA* and *vanB* resistance genes in enterococci isolated from cheese (Jurkovic et al., 2006). In another paper *vanA* gene was found in 37% of the dairy enterococci examined which, however, were all susceptible to vancomycin (Ribeiro et al., 2007). For the first time in Egypt, *E. faecalis* and *E. faecium* vancomycin-resistant strains were reported from food of animal origin by Hammad et al. (2015), which is in agreement with our finding.

The emergence of enterococci resistance to glycopeptides, including vancomycin and teicoplanin, in many of developed countries is attributed to a dual development that included clinical overuse and cross-resistance, following the use of avoparcin as an animal growth promoter (Koluman et al., 2009). Although enterococci are generally regarded as being intrinsically resistant to low levels of gentamicin, a high-level gentamicin resistance was detected in many dairy isolates (Giraffa, 2003; Hummel et al., 2007). All strains isolated in our study are susceptible to gentamicin. There are a few studies that investigate the spread of antibiotic resistance genes in camel's milk and it is very surprising to find vancomycin resistant *Enterococci* (VRE) in camel milk, knowing that this animal survives in areas far from the urban area. Camel pastoralists are nomadic, a matter which may explain the presence of enterococci isolates resistant to vancomycin, it is possible that these pastoralists carry strains resistant to vancomycin, as they are the only contact between urban centers and grazing areas where camels located. Or enterococci isolated from camel milk are intrinsically resistant to vancomycin. It is a hypothesis that needs to be verified. A hypothesis that could be confirmed by means of the additional molecular studies that are under way at our laboratory.

Conclusion

Camel milk is a very rich ecosystem that needs to be investigated. However, it has been long neglected, and has not had the opportunity to be a subject of large scale research. The results of the present study show that analysis of soluble whole-cell proteins can be used to discriminate between species of *Enterococcus* isolated from camel milk that are usually hard to differentiate by physiologic tests. Also there have been very few systematic studies to investigate acquired antibiotic resistance in enterococci of Camel's milk origin. We were surprised to discover in the camel milk the presence of vancomycin resistant enterococci, something that is scary. Fortunately, the incidence of penicillin, ampicillin and gentamicin resistance for all isolated strains was low, indicating that most of the strains tested did not acquire resistance determinants for these antibiotics. These results are about to be verified and validated by molecular techniques.

Conflict of Interests

The authors have not declared any conflict of interest.

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Toxin gene profile and antibiotic resistance of *Staphylococcus aureus* isolated from clinical and food samples in Egypt

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The antimicrobial sensitivity, toxin profiles, and *agr* genotyping of 70 *Staphylococcus aureus* isolates were determined. The evaluation of 10 antimicrobials showed that 88.5, 52.8, 40, and 25.7% of isolates were resistant to ampicillin, tetracycline, cefoxitin, and oxacillin, respectively. All isolates were sensitive to gentamicin. The prevalence of staphylococcal enterotoxin (SE) genes (*sea, seb, sec, sed, see, seg, sei,* and *seh*) and the toxic shock syndrome (*tsst-1*) gene were estimated by polymerase chain reaction (PCR); 95.7% of *S. aureus* carried the SE genes. The predominant gene was *sed* (75.7%), followed by *sea* and *tsst-1* (58.5%), then *see* and *sei* (51.4%). The *tsst-1* gene was found at a significantly higher rate among food isolates than clinical isolates (P= 0.003). The *agr* types were identified by multiplex PCR; *agr* II was more prevalent (58.5%) than *agr* I (25.7%) and *agr* III (20%).

Key words: *Staphylococcus aureus*, antibiotic resistance, enterotoxins, *agr* types.

INTRODUCTION

The Gram-positive bacterium *Staphylococcus aureus* is an opportunistic pathogen that is highly dangerous to human health. It can be identified in the anterior nares and skin of human (Kluytmans et al., 1997). This organism has the ability to cause infection of almost every tissue and organ system in human body, doing so by exporting a large array of virulence factors (surface proteins, enzymes and toxins) to the cell surface and extracellular environment of the human host (Vandecasteele et al., 2009). Both high virulence and rapid development of antimicrobial resistance contribute to the pathogenicity of *S. aureus* (Arvidson and Tegmark, 2001). Although pathogenesis of *S. aureus* is a multifactorial process that depends on multiple virulence factors' expression, some disease symptoms are particular to specific exotoxins including toxic shock syndrome toxin (tsst-1), enterotoxins (SEs) and exfoliative toxins (ETs) (Dinges et al., 2000).

S. aureus represents a major cause of food poisoning due to SEs produced by some strains. *S. aureus* produces large array of toxins (23 serologically different toxins) including staphylococcal enterotoxins (SEs) and

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staphylococcal enterotoxin-like proteins (SEls) (Hu and Nakane, 2014). SEs and SEls can be subdivided into classical (SEA to SEE) and new (SEG to SElX) types. SEs are considered the major cause of food poisoning, because they are resistant to heat and to proteolytic enzymes like trypsin, pepsin and renin; so, pass both cooking process and move through the gastrointestinal tract without losing their activity. They cause systemic effects such as high fever, abdominal cramps, vomiting, and diarrhea (Fernandez et al., 2006). These symptoms appear within few hours (1 to 6 h) of ingestion of food contaminated with very small quantities of SEs (20 ng to 1 µg/1 g of food) (Le Loir et al., 2003; Di Giannatale et al., 2011). Usually, infected individual recovers within one to two days, but some cases require hospitalization.

Food handlers are the primary source for contamination of foods with *S. aureus*. The foods that are commonly mentioned in staphylococcal food poisoning cases differ greatly from one country to another due to different eating and consumption habits. Contaminated raw meat is considered a major source of such type of food poisoning. The toxic dose of enterotoxigenic strains of *S. aureus* can be reached easily in ground meat before its consumption (Zargar et al., 2014).

The tsst-1 produced by some strain of the *S. aureus* is also a member of the superantigen family. It is responsible for toxic shock syndrome in all menstrual cases as well as 50% of non-menstrual ones. This toxin can affect immunity of infected individual causing it to diminish (Podbielska et al., 2011).

The objectives of this study were (i) to study the resistance profile of the isolates, (ii) to investigate the distribution of genes encoding SEs and tsst-1 toxin by polymerase chain reaction (PCR), and (iii) genotyping of isolates.

MATERIALS AND METHODS

Bacterial strains

During the period of January 2013 to January 2014, 370 samples were collected from clinical (70) and food (300) sources. Clinical samples (diabetic foot infections, wounds and abscesses) were collected from patients in Mansoura University hospitals, while food samples (75 ready to eat meat, 50 fresh meat, 75 mince beef and 100 beef burger) were collected from supermarkets and butchers shops distributed in Mansoura, Egypt. All samples were processed properly and evenly spread onto dried surfaces of Mannitol salt agar and Baird-Parker agar supplemented with egg-yolk tellurite emulsion, then incubated at 37°C for 24 to 48 h (Khalifa et al., 2014). Colonies exhibiting characteristic morphology of *S. aureus* were randomly selected and subjected to Gram stain, tests for catalase, coagulase enzymes and finally genotypic confirmation through PCR detection of *S. aureus* specific *nuc* gene (Brakstad et al., 1992). The experimental protocol conducted in the study was approved by the Ethical Committee of Faculty of Pharmacy, Mansoura University, Egypt, with code (2015-55).

Positive control strains DSM 19040 (*sec*, *see*, *tsst-1* and *agrI*), DSM 19041 (*sea*, *seb* and *sed*) and DSM 19048 (*seg*, *sei* and *agrIV*) were kindly provided by Dr. Paolo Moroni (University of Milan, Italy).

Antimicrobial susceptibility testing

Susceptibility of isolates to antimicrobials was determined by the Kirby-Bauer disc diffusion method using Mueller-Hinton agar plates (Oxoid). Nine antimicrobials were used including: ampicillin (AMP, 10 µg), oxacillin (OXA, 5 µg), cefoperazone (CFP, 30 µg), gentamicin (GEN, 10 µg), trimethoprim/ sulfamethoxazole (SXT, 1.25/23.75 µg), erythromycin (ERY, 15 µg), ciprofloxacin (CIP, 5 µg), tetracycline (TET, 30 µg) and cefoxitin (FOX, 30 µg) which was used for detection of methicillin resistant *S. aureus* (MRSA) (Hoseini Alfatemi et al., 2014).

All discs were purchased from Oxoid (U.K). After 18 to 24 h incubation at 37°C, the zone of inhibition was measured and isolates were recorded as sensitive or resistant according to the interpretation criteria of CLSI (2014).

DNA extraction

DNA was extracted by making a suspension of single colony from each isolate in 100 µl DNase/RNase-free water. Then, suspensions were maintained in a boiling water-bath for 10 min to lyse the cells, chilled on ice and centrifuged. Supernatants containing extracts of DNA were transferred to new eppendorf tubes and stored at -20°C for subsequent PCR amplification (Englen and Kelley, 2000).

PCR detection of toxins genes

The primers used for detection of SEs and *tsst-1* genes were specifically designed and ordered from Invitrogen (U.K). Primers sequences and expected amplicon sizes are shown in Table 1. The enterotoxin genes (*sea, seb, sec, sed, see, seg*, *seh* and *sei*) and *tsst-1* gene were detected using 25 µl reaction: 12.5 µl Dream Taq™ Green PCR Master Mix (2X) (Fermentas, U.K), 1 µl of forward primer (10 µM), 1 µl of reverse primer (10 µM), 3 µl of the DNA extract and 7.5 µl of nuclease free water. The PCR reactions were started with initial denaturation step at 95°C for 5 min, followed by 40 cycles of denaturation at 95°C for 30 s, annealing at 54°C for *sea, seb, sec, sed, see, seg*, *seh* and *tsst-1* and 56°C for *sei* for 30 s and extension at 72°C for 1 min and final extension at 72°C for 5 min. Positive control for the PCR-based assays were included with each primer set. For negative control, ddH_2O was used instead of DNA extract.

Following amplification reactions, the PCR products were analyzed via electrophoresis in a 2% agarose gel (stained with ethidium bromide) at 100 V for 45 min in 1 X Tris-borate-EDTA buffer and illuminated under UV transilluminator and photographed. The Gene Ruler™ 100 bp Plus DNA Ladder (MBI Fermentas, St. Leon-Rot, Germany) was used as a DNA size marker. A reference strain for the *seh* gene was not available; therefore, the presence of a band at the expected product size was considered a positive result.

agr genotyping

The *agr* allele types (I to IV) were detected by multiplex PCR as described previously (Gilot et al., 2002; Xie et al., 2011). Positive control strains for *agr* allele types (I and IV) were included in each run of PCR. Reference strains for *agrII* and *agrIII* were not available, so the presence of bands at the expected product size was considered a positive result.

Electrophoresis agarose gels of toxins and *agr* groups were analyzed visually and scored using a binary code that was analyzed using DendoUPGMA. A dendogram was constructed applying the unweighted pair group method with arithmetic mean

Table 1. Primers sequences used for PCR amplification of the tested genes.

Gene	Sequence	Amplicon size (bp)	Reference
sea	F 5'-AGCTTGTATGTATGGTGGTGT-3' R 5'-ACGTCTTGCTTGAAGATCCA-3'	172	This study
seb	F 5'-AGGACACTAAGTTAGGGAAT-3' R 5'-CTCAGTTACACCACCATACA-3'	200	This study
sec	F 5'-TGTAGGTAAAGTTACAGGTGGT-3' R 5'-TGTCTAGTTCTTGAGCTGTTAC-3'	182	This study
sed	F 5'-GTTTGATTCTTCTGATGGGTCT-3' R 5-GAAGGTGCTCTGTGGATAATG-3'	119	This study
see	F 5'-GATCTTCAGGCAAGGCATTATT-3' R 5'-TAACTTACCGTGGACCCTTC-3'	122	This study
seg	F 5'-CGACAATAGACAATCACTTGGA-3' R 5'-TCCAGATTCAAATGCAGAACC-3'	142	This study
seh	F 5'-TGCGAAAGCAGAAGATTTACAC-3' R 5'-TCATTGCCACTATCACCTTGA-3'	165	This study
sei	F 5'-GGCAGTCCATCTCCTGTATAA-3' R 5'-AACACTGGTAAAGGCAAAGAAT-3'	134	This study
tsst-1	F 5'-CGTAAGCCCTTTGTTGCTTG-3' R 5'-TGTCAGACCCACTACTATACCA-3'	143	This study
nuc	F 5'-GCGATTGATGGTGATACGGTI-3' R 5'-AGCCAAGCCTTGACGAACTAAAGC-3'	267	Brakstad et al. (1992)

F: forward; R: reverse.

(UPGMA), using the Jaccard coefficient, with the program DendoUPGMA [Univertat Rovirai Virgili (URV), Tarragona, Spain].

Statistical analysis

Data analysis was performed using GraphPad Instat 3.10, chi-square test was used. The difference is considered significant if the P value ≤0.05.

RESULTS

Isolation of *S. aureus*

A total of 70 strains were separated and identified as *S. aureus* [35 strains out of 300 food samples (11.7%) and another 35 strains out of 70 clinical samples (50%)]. The genotypic identification was in accordance with the phenotypic characterization results.

Antimicrobial susceptibility test

The resistance percentage of *S. aureus* isolates to different antimicrobials is shown in Table 2. No significant association was detected between isolates sources and antimicrobials resistance (p >0.05 for each antimicrobial). A multidrug resistance phenotype (resistance to ≥3 classes of antimicrobial agents) was exhibited by 12 (17.1%) isolates; 7 of them were derived from food. Forty percent of the tested isolates were found to be methicillin resistant (MRSA). Methicillin resistance was equally distributed among the food and clinical isolates. Resistance to large number of tested antimicrobials (3 to 5 and 6 to 8 antimicrobials) was mostly associated with

Table 2. Resistance percent of *S. aureus* isolates to the tested antimicrobial agents.

Source	Resistance (%)								
	AMP	CFP	CIP	ERY	GEN	TET	STX	OXA	FOX
Food Isolates	91.42	11.42	0	11.42	0	42.85	5.71	25.71	40
Clinical Isolates	85.71	8.57	8.57	11.42	0	62.85	5.71	25.71	40

AMP: Ampicillin; TET: tetracycline; FOX; cefoxitin; OXA; oxacillin; E; erythromycin; CFP; cefoperazone; SXT: trimethoprim/sulfamethoxazole; CIP: ciprofloxacin; GEN: gentamicin.

Table 3. Antibiotic resistance patterns of the tested *S. aureus* isolates.

Antibiotype	Number of food isolates	Number of clinical isolates	Resistance pattern
A1	10	6	AMP
A2	1	3	TET
A3	5	6	AMP, TET
A4	3	1	AMP, FOX
A5	1	1	AMP,CFP
A6	-	1	AMP, E
A7	-	1	AMP, OXA
A8	2	1	AMP, CIP
A9	1	-	AMP,CFP, TET
A10	-	1	AMP, CFP, FOX
A11	2	-	AMP, OXA, FOX
A12	2	-	AMP, TET, OXA
A13	2	4	AMP, TET, FOX
A14	-	1	ERY,TET, FOX
A15	1	-	AMP, ERY, TET, FOX
A16	1	-	AMP, TET, SXT, FOX
A17	1	4	AMP, TET, OXA, FOX
A18	1	-	AMP, CFP, OXA, FOX
A19	1	1	AMP, ERY, TET, OXA, FOX
A20	-	1	AMP, CFP, CIP, TET, OXA, FOX
A21	-	1	AMP, CFP, TET, SXT, OXA, FOX
A22	-	1	AMP, CIP, ERY, SXT,OXA, FOX
A23	1	-	AMP, CFP, ERY, TET, SXT, OXA, FOX
A24	-	1	Sensitive to all

AMP: Ampicillin; TET: tetracycline; FOX; cefoxitin; OXA; oxacillin; E; erythromycin; CFP; cefoperazone; SXT: trimethoprim/sulfamethoxazole; CIP: ciprofloxacin.

MRSA regardless of the source of isolates (P<0.0001).

There was a wide range of resistance patterns among isolates, as 24 different patterns were observed (Table 3). Nine patterns were found among isolates of both origins (A1, A2, A3, A4, A5, A8, A13, A17 and A19). The most common resistance pattern was A1 that was exhibited by 22.8% of isolates showing resistance to ampicillin only.

Detection of SE and *tsst-1* genes

The PCR amplification of toxin genes showed that one or more toxin genes were carried by 95.7% of the isolates (Figure 1). The *sed* gene was the most abundant toxin gene among isolates with a rate of 75.7%, while *seh* gene was the least frequently detected one (11.4%) as shown in Table 4. No significant differences were found between isolates from food and clinical origins except for the *tsst-1* gene where it was significantly higher in the food isolates (P=0.003). Through analysis of PCR results, forty nine toxin genes patterns were detected (Table 5). Seven toxicity patterns were exhibited by both food and clinical isolates (T1, T2, T8, T14, T37, T41, and T45). While 19 toxicity patterns were found in food isolates

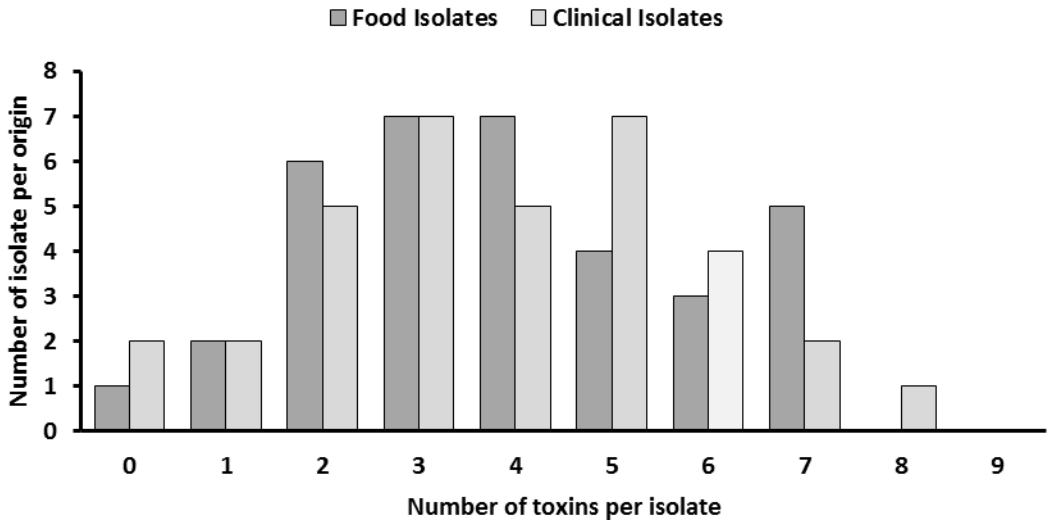


Figure 1. Number of toxin genes per isolate in clinical and food isolates.

Table 4. Prevalence of toxin genes in *S. aureus* isolates.

Toxin gene	Total (%)	Food isolates [n=35 (%)]	Clinical isolates [n=35 (%)]	P value
sea	41 (58.5)	20 (57.1)	21 (60)	1.000
seb	18 (25.7)	8 (22.8)	10 (28.5)	0.785
sec	26(37.1)	15 (42.8)	11 (31.4)	0.458
sed	53 (75.7)	28 (80)	25 (71.4)	0.578
see	36 (51.4)	19 (54.2)	17 (48.5)	0.811
seg	11 (15.7)	4 (11.4)	7 (20)	0.513
seh	8(11.4)	1 (2.8)	7 (20)	0.054
sei	36 (51.4)	14 (40)	22 (62.8)	0.093
tsst-1	41 (58.5)	27 (77.1)	14 (40)	0.003**

Table 5. Toxin genes patterns of *S. aureus* isolates.

Pattern No.	Toxin profile	Food isolates	Clinical isolates
T1	no toxin genes	1	2
T2	*sed*	1	1
T3	*seg*	-	1
T4	*tsst*	1	-
T5	*sea, sed*	2	-
T6	*sea, see*	-	1
T7	*sea, tsst*	2	-
T8	*seb, sed*	1	1
T9	*sec, sei*	-	2
T10	*sed, sei*	-	1
T11	*sed, tsst*	1	-
T12	*sea, sec, sed*	-	2
T13	*sea, sec, tsst*	1	-
T14	*sea, sed, see*	1	1
T15	*sea, see, sei*	0	1
T16	*seb, sed, tsst*	2	-

Table 5. Contd.

T17	*sec, sed, see*	-	1
T18	*sec, sed, sei*	-	1
T19	*sec, sed, tsst*	-	1
T20	*sec, see, tsst*	1	-
T21	*sed, see, tsst*	2	-
T22	*sea, seb, sei, tsst*	-	1
T23	*sea, sec, sed, see*	1	-
T24	*sea, sec, see, tsst*	1	-
T25	*sea, sed, see, sei*	-	2
T26	*sea, sed, see, seh*	-	1
T27	*sea, sed, see, tsst*	1	-
T28	*sea, seh, sei, tsst*	-	1
T29	*seb, sed, see, tsst*	1	-
T30	*seb, sed, sei, tsst*	2	-
T31	*sec, sed, sei, tsst*	1	-
T32	*sea, seb, sed, see, tsst*	-	1
T33	*sea, seb, seh, sei, tsst*	-	1
T34	*sea, sec, sed, see, seg*	1	-
T35	*sea, sec, sed, seh, sei*	-	1
T36	*sea, sed, see, sei, tsst*	2	-
T37	*sea, sed, seg, sei, tsst*	-	1
T38	*seb, sed, seg, seh, sei*	-	1
T39	*sec, sed, see, sei, tsst*	1	1
T40	*sed, see, seg, sei, tsst*	-	1
T41	*sea, seb, sed, see, seg, sei*	-	1
T42	*sea, seb, sed, see, sei, tsst*	-	2
T43	*sea, sec, sed, see, sei, tsst*	3	1
T44	*sea, seb, sec, sed, see, sei, tsst*	1	-
T45	*sea, seb, sec, sed, seg, sei, tsst*	1	-
T46	*sea, seb, sed, see, seg, sei, tsst*	-	1
T47	*sea, sec, sed, see, seh, sei, tsst*	1	1
T48	*sea, sec, sed, see, seg, sei, tsst*	2	-
T49	*sea, seb, sed, see, seg, seh, sei, tsst*	-	1

only. Another 23 toxicity patterns were found in clinical isolates.

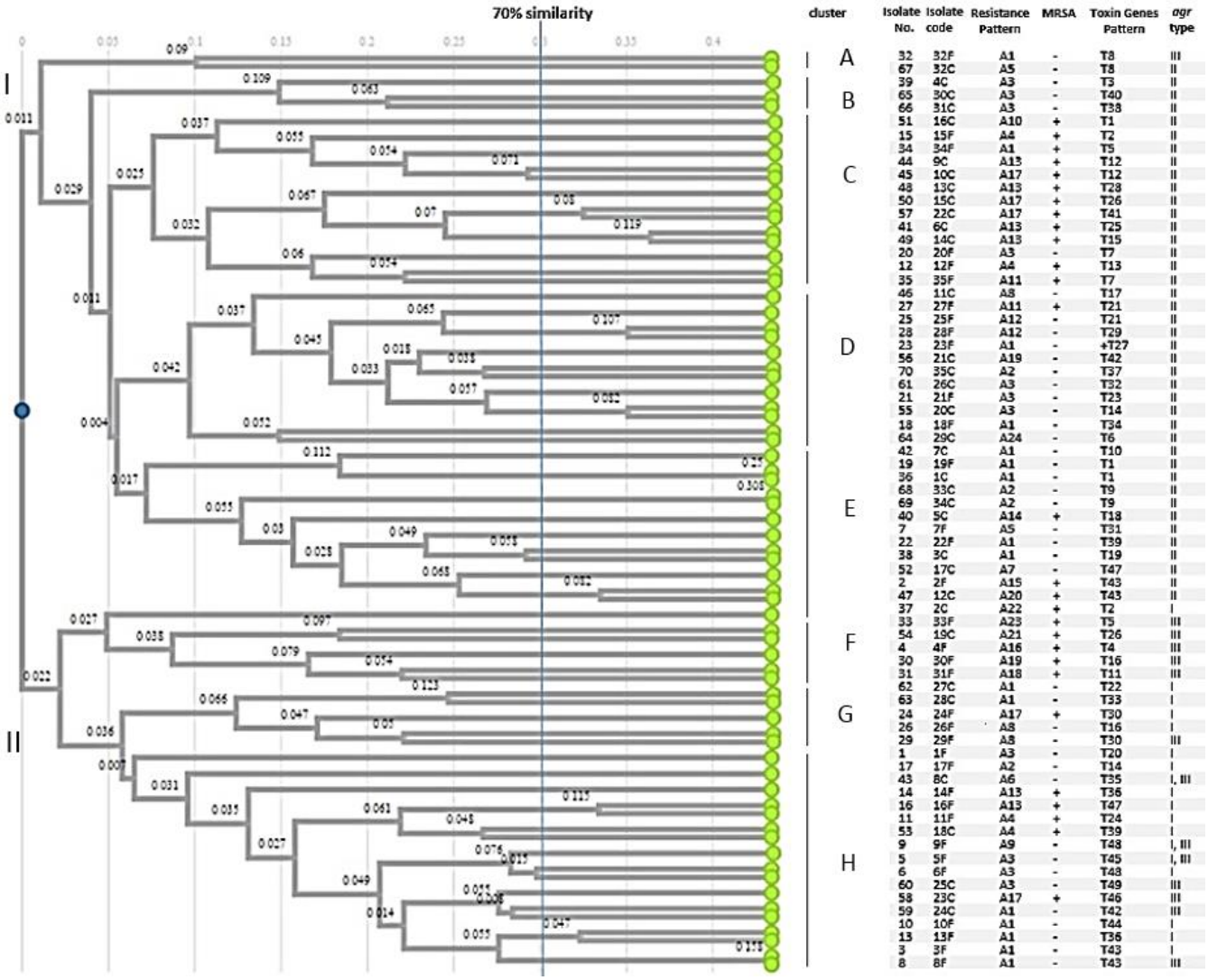
agr typing

The majority of our isolates (58.5%) belonged to *agr* type II, followed by *agr* type I (25.7%) and *agr* group III (20%). None was positive for *agr* type IV.

In the 70 *S. aureus* isolates, the constructed dendrogram showed two large clusters (Figure 2). Cluster I comprises 42 isolates of *agr* II type (except one isolate of *agr* III type). It was subdivided into five smaller clusters (A to E). The second large cluster II contained 28 isolates of either *agr* I, *agr* III or mixed *agr* I/III which was subdivided into three smaller clusters (F to H). The number of isolates in each small cluster varied greatly. Whereas cluster A contained only two isolates, clusters H comprised 17 isolates. Each of clusters E and H had three pairs of isolates that showed 100% identity. Moreover, clusters C, D and H contained 2 mini clusters each and only one mini cluster in cluster E. These mini clusters showed more than 70% similarity.

DISCUSSION

In this study, 70 *S. aureus* strains were isolated from 370 samples (18.9%). During sampling, high prevalence rate of *S. aureus* was expected originating from hands of food handlers, because of poor hygiene processes employed, but only 11.7% of food samples were positive for *S. aureus*. On the other hand, 50% of clinical samples were positive for *S. aureus* which may indicate the ease of



Isolate No.	Isolate code	Resistance Pattern	MRSA	Toxin Genes Pattern	*agr* type
32	32F	A1	-	T8	III
67	32C	A5	-	T8	II
39	4C	A3	-	T3	II
65	30C	A3	-	T40	II
66	31C	A3	-	T38	II
51	16C	A10	+	T1	II
15	15F	A4	+	T2	II
34	34F	A1	+	T5	II
44	9C	A13	+	T12	II
45	10C	A17	+	T12	II
48	13C	A13	+	T28	II
50	15C	A17	+	T26	II
57	22C	A17	+	T41	II
41	6C	A13	+	T25	II
49	14C	A13	+	T15	II
20	20F	A3	-	T7	II
12	12F	A4	+	T13	II
35	35F	A11	+	T7	II
46	11C	A8	-	T17	II
27	27F	A11	+	T21	II
25	25F	A12	-	T21	II
28	28F	A12	-	T29	II
23	23F	A1	-	+T27	II
56	21C	A19	-	T42	II
70	35C	A2	-	T37	II
61	26C	A3	-	T32	II
21	21F	A3	-	T23	II
55	20C	A3	-	T14	II
18	18F	A1	-	T34	II
64	29C	A24	-	T6	II
42	7C	A1	-	T10	II
19	19F	A1	-	T1	II
36	1C	A1	-	T1	II
68	33C	A2	-	T9	II
69	34C	A2	-	T9	II
40	5C	A14	+	T18	II
7	7F	A5	-	T31	II
22	22F	A1	-	T39	II
38	3C	A1	-	T19	II
52	17C	A7	-	T47	II
2	2F	A15	+	T43	II
47	12C	A20	+	T43	II
37	2C	A22	+	T2	I
33	33F	A23	+	T5	III
54	19C	A21	+	T26	III
4	4F	A16	+	T4	III
30	30F	A19	+	T16	III
31	31F	A18	+	T11	III
62	27C	A1	-	T22	I
63	28C	A1	-	T33	I
24	24F	A17	+	T30	I
26	26F	A8	-	T16	I
29	29F	A8	-	T30	III
1	1F	A3	-	T20	I
17	17F	A2	-	T14	I
43	8C	A6	-	T35	I, III
14	14F	A13	+	T36	I
16	16F	A13	+	T47	I
11	11F	A4	+	T24	I
53	18C	A4	+	T39	I
9	9F	A9	-	T48	I, III
5	5F	A3	-	T45	I, III
6	6F	A3	-	T48	I
60	25C	A3	-	T49	III
58	23C	A17	+	T46	III
59	24C	A1	-	T42	III
10	10F	A1	-	T44	I
13	13F	A1	-	T36	I
3	3F	A1	-	T43	I
8	8F	A1	-	T43	III

Figure 2. Dendrogram of genotypic relationship of *S. aureus* isolates. Each pattern listes the sample sources, antimicrobial resistance pattern, toxin gene contents and *agr* types of the isolates. C: Clinical; F: food.

transmission of this microbe between patients. Similar finding were reported previously on prevalence of *S. aureus* in food (Normanno et al., 2007) and clinical samples (Ahmed et al., 2014).

Ninety five percent of tested isolates showed antimicrobial resistance to at least one antimicrobial; this is similar to previous studies (Normanno et al., 2007; Rhee and Woo, 2010; Aydin et al., 2011). Resistant to ampicillin was 88.5% which is consistent with previous reports that showed high resistance rates of *S. aureus* (>90%) to ampicillin (Pereira et al., 2009; Daka and Yihdego, 2012).

The prevalence of MRSA has increased globally (Jones et al., 2003). In this study, the rate of MRSA occurrence was found to be 40%; distributed equally between food and clinical isolates. Pereira et al. (2009) found MRSA among food strains in an approximately the same percent (Pereira et al., 2009). Lee do et al. (2008) reported a higher occurrence rate of MRSA among *Staphylococcus* isolated from clinical samples and raw meats (Lee do et al., 2008).

Resistance profile recorded in our study revealed that multidrug resistance to at least 3 antimicrobials was associated with MRSA. These results were in accordance with two previous studies in 2009 (Al-Khulaifi Manal et al., 2009; Galkowska et al., 2009). An explanation is that all MRSA strains have SCC*mec* (staphylococcal cassette chromosome *mec*) which act as reservoir for the different Staphylococcal genes, especially those encoding resistance (Al-Khulaifi Manal et al., 2009). Also, 24 resistance patterns were demonstrated by isolates. Ampicillin and ampicillin/tetracyclin resistance was the

most recorded pattern exhibited by isolates (22.8 and 15.7%, respectively).

Previous studies reported that microbial isolates from food acquired resistance against most antibiotics (Valsangiacomo et al., 2000; Yucel et al., 2005). This observation was already recorded in our study where strains from food samples showed high resistance levels to antimicrobials similar to clinical ones. This may be explained by the use of antimicrobials in food production and veterinary medicine resulting in increased resistance to antimicrobials used for human therapy (Yucel et al., 2011).

PCR-based detection of enterotoxins *sea-see*, *seg-sei* and *tsst-1* genes showed that one or more toxin genes were carried by 95.7% of isolates. Similar results were reported previously (Pu et al., 2011; Xie et al., 2011). In our study, *sed* gene was the most prevalent toxin gene among isolates (75.7%). This finding agrees in part with Normanno et al. (2007) who reported prevalence of *sed* gene compared to other SEs detected. Several studies reported higher prevalence to other toxin genes such as *sea*, *sec* and *tsst-1* in the tested isolates (Klotz et al., 2003; Chiang et al., 2008; Pumtang-on et al., 2008). The difference in prevalence of superantigenic toxin genes may be attributed to that enterotoxin genes as well as tsst-1 gene are mostly found on mobile genetic element (Balaban and Rasooly, 2000) which facilitate its transfer among the same or different species of bacteria (Varshney et al., 2009; Malachowa and DeLeo, 2010). So, prevalence of such toxins may depend on the possibility of its transfer between isolates. In accordance with Alibayov et al. (2014) *seh* gene was the least prevalent gene detected.

The coexistence of *seg* and *sei* genes was recorded in only 10% of our isolates. This finding was partly consistent with Mashouf et al. (2015) where *seg* and *sei* genes were detected separately. Several authors have reported the presence of *seg* and *sei* genes individually or in different combinations with other SEs genes (Cremonesi et al., 2007; Zouharova and Rysanek, 2008; Arcuri et al., 2010). The prevalence of classical enterotoxin genes (*sea-see*) in the tested isolates was 92.85%, while that of the new enterotoxin genes was 55.7%. This result agrees with the results of the previous studies (Rall et al., 2008; Wu et al., 2010; Mashouf et al., 2015).

The *tsst-1* gene was detected in 58.5% of isolates. A similar result was reported in a study of staphylococcal food-poisoning outbreaks in Taiwan (Chiang et al., 2008). No significant differences were found between *S. aureus* isolates of food and clinical origins except for the *tsst-1* gene where it was significantly higher in the food isolates than in the clinical ones (P=0.003). A previous study reported higher prevalence of *tsst-1* gene in food isolates than human and animal isolates (Adesiyun et al., 1992). On analysis of toxin gene profile results, 94% of enterotoxigenic isolates were found to harbor more than one toxin gene. This percentage is higher than data reported previously (Becker et al., 2003; Udo et al., 2009). Forty nine toxin patterns were detected in our isolates. This finding was in accordance with Xie et al. (2011) who found 47 toxin patterns. The high diversity of toxin gene profiles of *S. aureus* can be used in addition to the present genotyping methods to achieve high discrimination between isolates.

The expression of many virulence genes such as exotoxins and capsular polysaccharides type 5 and 8 during *S. aureus* infections is influenced by *agr* locus (Luong et al., 2002). This is considered a mechanism for isolating bacterial populations and a basis for species subdividing (Robinson et al., 2005).

During this study, the prevalence of *agr* specificity groups have been investigated using multiplex PCR which revealed that all analyzed strains harbored *agr* gene based on the amplicon size differences. The results showed that the *agr* group II was the most prominent detected in 58.5% of isolates; followed by *agr* group I (25.7%) and *agr* group III (20%). This was in accordance with data supplied by Ayepola (2012) who showed that 82% of *S. aureus* isolated from clinical sources belonged to *agr* II.

On the other hands, an earlier study showed that *agr* I was the most prevalent (van Leeuwen et al., 2000). Two food and one clinical isolates showed mixed *agr* type as they carried both *agr* I and III. Mixed *agr* I/IV was reported by previous studies (Goerke et al., 2005; Robinson et al., 2005). Further investigations are required for more characterization of these isolates.

No significant correlation was found between *agr* type and the isolation sources ($P > 0.05$) as prevalence of a particular *agr* group was not found among either food or clinical isolates. Concerning toxin genes, *agr* I was significantly associated with *sei* and *tsst-1* genes (P= 0.0033 and 0.0005, respectively). Besides that, *agr* II was correlated with *sed* and *see* genes (P =0.0003, 0.0213, respectively). In accordance with Xie et al. (2011), no correlation was found between *agr* types and toxin gene profile of the *S. aureus* strains.

Hierarchical clustering of isolates on the basis of *agr* type, resistance pattern and toxin genes pattern results in two large clusters. These clusters contained isolates from both sources, but the majority of clinical isolates (74.2%) was found in cluster I. Isolates of *agr* I/III type were grouped in one cluster (H). Although three pairs of isolates showed 100% similarity [(33c, 34c), (3f, 8f) and (19f, 1c)], they were isolated from different locations. This result suggests the need for other typing techniques to analyze the genetic background of these isolates.

Conclusions

High resistance levels were detected in *S. aureus* isolates either from food or clinical sources in Egypt. This result is an alert of the growing problem of bacterial resistance

mostly due to the improper use of antibiotics. Although incidence of *S. aureus* isolates in food samples was not high, these isolates were highly enterotoxigenic. Moreover, toxin gene profile analysis can be used in genotyping of enterotoxigenic strains as it allows a better discrimination for isolates than *agr* typing which is limited only to four groups.

Conflict of Interests

The authors have not declared any conflict of interests.

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Full Length Research Paper

Morphoagronomic and productive traits of RR® soybean due to inoculation via *Azospirillum brasilense* groove

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In the last decades, the Brazilian soy productive chain has passed through a transformation process in which the yield, efficiency, profitability, economic and environmental sustainability are very important issues. In this context, the introduction of microorganisms has provided an increase in grains yield. The objective of this study was to evaluate the inoculation of *Azospirillum brasilense* associated with *Bradyrhizobium japonicum* in agronomic traits and the soybean productivity. The experiment was conducted in randomized blocks in 4 × 2 factorial, with four cultivars (Anta 82 RR®, BRS Favorita RR®, BRS 780 RR®, BRS 820 RR®) and two treatments with *A. brasilense* (inoculated and non-inoculated) with three replications in two growing seasons. The following traits were evaluated: plant height, shoot dry biomass, chlorophyll content, leaf nitrogen content at flowering; and at harvest, the plant height, the insertion of the first pod, number of pods per plant, number of grains per pod, thousand-grain weight , grain yield, and grain harvest index were evaluated. There was a significant effect of growing seasons and cultivars in an isolated way and their interaction in most traits. The conditions in which the study was conducted, with or without inoculation of *A. brasilense* associated with *B. japonicum* do not affect the agronomic traits and grain yield in RR® soybean cultivars.

Key words: *Glycine Max* (L.) Merrill, growth promoting bacteria, rhyzobium.

INTRODUCTION

Soybean [*Glycine max* L. Merrill] is an oilseed of great economic importance in the national and international market due to the high levels of protein and oil in their seeds/grains (Lima et al., 2015). The cultivation has been widely studied due to its high nutritional value and great consumer market. FAO (2013) reported that the soybean produced is enough to provide one-third of the global need of food proteins, since it is completely intended for

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Table 1. Chemical composition of a typical oxisol (0.0-0.2 m) before the experiment installation, Lavras-MG, Brazil, for the 2013/2014 and 2014/2015 growing seasons.

Growing season	pH	Ca^{2+}	Mg^{2+}	Al^{3+}	$H^{+}+Al^{3+}$	SB	CEC	P	K	MO	V
	H_2O	$cmol_c\ dm^{-3}$						$mg\ dm^{-3}$		$dag\ kg^{-1}$	%
2013/14	6.4	5.0	1.4	0	2.9	6.7	9.6	11.46	118	3.41	69.82
2014/15	6.2	3.80	0.80	0	0.9	4.8	5.7	20.83	92	2.23	83.52

H + Al: Potential acidity; SB: sum of bases; CEC: cation-exchange capacity at pH 7.0; MO: organic matter; V: base saturation.

human consumption.

In this scenario, Brazil is the second largest world producer and exporter of soybeans, with 31,902,400 ha^{-1} of sown area, with an average grain yield of 3011 kg ha^{-1} in the 2014/2015 harvest (CONAB, 2015). The Brazilian soy productive chain has undergone modernization processes, which provide the increase in grain yield (Zuffo et al., 2015a).

The introduction of *Bradyrhizobium*, which performs the biological nitrogen fixation (BNF), was one of the major drivers for large-scale soy cultivation in Brazil (Zuffo et al., 2015b). Therefore, the technological advances in soil microbiology area are important for the viability of soybean cultivation. Besides these bacteria, the soil is an ecosystem that has a great variety of plant growth promoting bacteria (PGPBs) that can be free-living or associated with plant roots.

For Silveira and Freitas (2007), PGPBs constitute any bacteria that have beneficial effects on the growth of one or more vegetal species, except for the rhizobia that despite the beneficial relation to vegetal growth, is the result of a symbiotic relationship. In the literature, the most studied diazotrophs bacteria as associative PGPBs, belong to the genus *Azospirillum*. Mainly due to its use as inoculants commercialized in Brazil with a recommendation for the grasses, Hungria (2011) while using the *Azospirillum,* noted increases of 31 and 26% for grain yield in wheat and corn crops, respectively, but with supply of part of the nitrogen required by the plant by the mineral fertilizer.

For Araujo (2008), the *Azospirillum* has the following advantages: the bacteria are endophytic, that is, penetrates the roots of plants; presents antagonism to pathogens; produces phytohormones; it is not very sensitive to temperature variations; and occurs in all kinds of soil and climate. Among the plant hormones, research has demonstrated the ability of *Azospirillum brasilense* in producing auxin, gibberellins, and citocianinas under "*in vitro*" conditions (Masciarelli et al., 2013).

The use of inoculation with *A. brasilense* in leguminous plants has been studied; however, the effects are still contradictory. Reports presented by Bárbaro et al. (2009), Hungria et al. (2013) and Hungria et al. (2015) show influences on the agronomic traits of soybean crop, but the results checked by Gitti et al. (2012) and Zuffo et al. (2015a), do not support the authors mentioned earlier.

Therefore, the objective of this study was to evaluate the *A. brasilense* inoculation associated with *Bradyrhizobium japonicum* and its influence on agronomic traits and soybean yield.

MATERIALS AND METHODS

The experiment was conducted in the 2013/2014 and 2014/2015 growing seasons, in Lavras - MG, at the Scientific and Technological Development Center of Agriculture – Muquem Farm, located at latitude 21°12'S, 44°58'W longitude and altitude of 918 m in soil classified as Dystroferric Red Latosol - Oxisol, with clayey texture, with the following textural values: Clay: 640 g kg^{-1}; Silt: 200 g kg^{-1}; Sand: 160 g kg^{-1}. The chemical composition of the experimental area soil is shown in Table 1.

The climate is Cwa according to the Köppen classification, with average annual temperature of 19.3°C and normal annual rainfall of 1,530 mm (Dantas et al., 2007). Climatic data during the experiments were collected at the weather station of the National Institute of Meteorology (INMET) located at the Federal University of Lavras-UFLA and are presented in Figure 1.

The experimental design was a randomized block, arranged in a 4 × 2 factorial, with four cultivars (Anta 82 RR®, BRS Favorita RR®, BRS 780 RR®, BRS 820 RR®) and two treatments with *A. brasilense* (inoculated and non-inoculated) with three replications. Each plot consisted of four sowing lines of 5 m in length spaced in 0.50 m, and the area of each plot was of 10 m^2 (5 m × 2 m). The two central rows were considered as the useful area.

The sowing was carried in November of each growing season. Fertilization consisted of 350 kg ha^{-1} of the N-P_2O_5-K_2O (02-30-20) formulated, applied via groove. *B. japonicum* (Brad) and *A. brasilense* (Azos) bacteria were inoculated via groove after soybean sowing. The dosage of *B. japonicum* was 18 ml kg^{-1} of seed - SEMIA 5079 and 5080 strains, containing 10.8×10^6 CFU/seed of the inoculant Nitragin Cell Tech HC® (3×10^9 CFU/ml). *A. brasilense* used the dosage of 6 ml kg^{-1} of seed - AbV5 AbV6 strains, containing 24×10^4 CFU/seed of the inoculant Azo® (1×10^8 CFU/ml).

The microorganisms inoculation was carried out using a motorized backpack sprayer, coupled to the bar with four spray tips XR 110.02, applying spray volume equivalent to 150 L ha^{-1}. First, there was inoculated *B. japonicum* and then the *A. brasilense*.

At the beginning of flowering (R_1) plant height with assistance of a millimeter rule, shoot dry biomass using a forced air circulation oven at 60°C for 72 h until constant weight, with posterior plant residues weighting were determined. The collection of leaves (third trifoliate from top to bottom) was also held, then washed in deionized water and placed with the shoot dry biomass for drying. The dried leaves were ground in a Wiley mill. Chemical analysis of leaf tissue of macro and micronutrients were conducted according to the methodology described by Sarruge and Haag (1974); leaf chlorophyll content using chlorophyll portable model SPAD 502 Plus® by measuring 3 points in each trifoliate leaf in different parts

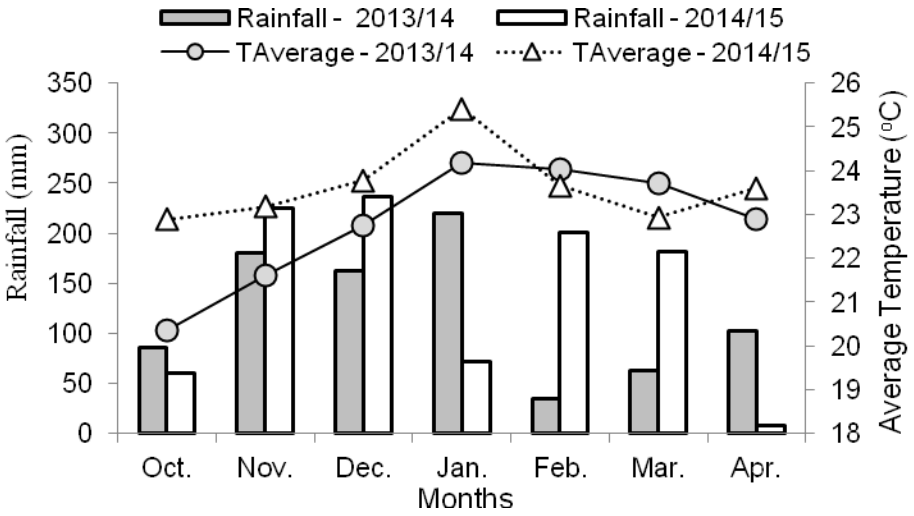

Figure 1. Monthly means for rainfall and air temperature in Lavras-MG, Brazil, for 2013/2014 and 2014/2015 growing seasons, during the experiment evaluations (National Institute of Meteorology (INMET).

on the same leave, always in the leaf blade between the nerves in the third trefoil from top to bottom.

At harvest, the following traits were obtained: plant height and insertion of the first pod with millimeter ruler assistance; then the collection of five plants per plot was held to assess the number of pods per plant and number of grains per pod through manual counting; thousand grain weight according to the methodology described in Brasil (2009); grain yield standardized to grain moisture of 13% in Kg/ha^{-1}. The grain harvest index (GHI) was also determined in the following way: GHI = grain yield/grains productivity + straw.

Analyses of individual and joint variances were performed adopting the statistical model and an analysis procedure similar to that presented by Ramalho et al. (2012). The means were grouped by the Scott-Knott test (1974). Statistical analysis was performed with assistance of statistical package SISVAR® (Ferreira, 2011).

RESULTS AND DISCUSSION

Except for plant height on flowering, nitrogen amount on leaf tissues and height of insertion of the first pod for the growing season were found that to have a significant (P≤0.01) influence on the studied variables (Tables 2 and 3). This results are in accordance with Felisberto et al. (2015) who also observed differences on soy agronomic variables according to the crop year.

The number of grain per pod and the grain harvest index were not significantly influencied by cultivars (Table 2). Soares et al. (2015a) and Felisberto et al. (2015) observed an effect of cultivars on the agronomic traits evaluated. The authors affirm that it was expected, since cultivars have different genetic background, growth habit, maturation group and other characteristics providing variations.

Regarding the inoculation with *A. brasilense*, no statistical differences on the evaluated characteristics were observed. Zuffo et al. (2015b) also observed no differences with the inoculation of soybean seeds with only *A. brasilense* or co-inoculated with *B. japonicum* on the plant height, number of trefoil, shoot dry biomass and roots, dry mass of nodules, root volume, chlorophyll content and leaf nitrogen, in accordance with the results of this study.

When the interaction between cultivar × growing season was studied, no statistical differences were observed for the first pod insertion height, yield and production of grain components (Tables 2 and 3). The interaction between cultivar × growing season was also verified by Soares et al. (2015b), indicating that the responses, considering the environmental variations, were not the same for the evaluated cultivars. Since the growing seasons presented differences for rainfall occurrence and temperatures, variations that are unpredictable were expected and the researcher do not have control.

For the interactions between growing season × *A. brasilense*, cultivar × *A. brasilense* and growing season × cultivar × *A.brasilense,* no statistical differences were observed (Tables 2 and 3). Therefore, it can be inferred that the inoculation with *A. brasilense* have no relation with cultivar and growing season. This fact can be explained by the absence of variability of the “variation cause” (*A. brasilense*).

The highest values for shoot dry biomass, chlorophyll content, plant height on harvest, number of pods per plant, number of grains per pod, grain yield and harvest index were obtained during the 2013/2014 growing season. It was due to the climatic conditions (Figure 1), mainly during January flowering, when a high pluviometric index was observed for the 2013/2014 growing season.

The highest values for number of pods per plant and

Table 2. Analysis of variance and means for plant height at flowering (PHF), shoot dry biomass (SDB), chlorophyll content (CLC) and N (NC) in leaves tissues of RR® soy on flowering stage (R_1), obtained with inoculation and non-inoculation of *Azospirillum brasilense* with *Bradyrhizobium japonicum* in RR® soy cultivars, to 2013/14 and 2014/15 growing seasons. Lavras-MG, Brazil.

Cause of variation	DF	ANOVA (QM)[1]			
		PHF	SDB	CLC	NC
		cm		unity	
Block (Bl)	2	5.17	113.31	6.86	5.07
Year (Yr)	1	9.72^{ns}	15330.20**	171.25**	10.45^{ns}
Cultivar (C)	3	255.90**	166.77*	19.19*	49.88**
Azospirillum (Az)	1	9.01^{ns}	121.28^{ns}	0.14^{ns}	7.36^{ns}
An × C	3	36.72^{ns}	95.70^{ns}	3.48^{ns}	19.26^{ns}
An × Az	1	1.47^{ns}	68.45^{ns}	6.35^{ns}	20.28^{ns}
C × Az	3	45.59^{ns}	81.31^{ns}	2.03^{ns}	5.33^{ns}
Yr × C × Az	3	5.04^{ns}	112.54^{ns}	2.07^{ns}	0.38^{ns}
Bl × Yr	2	22.55^{ns}	336.30**	0.01^{ns}	0.66^{ns}
Error	28	16.12	36.76	5.50	7.29
Mean	-	53.47	57.88	39.54	43.82
CV (%)	-	7.51	10.47	5.93	5.98
Factors			**Means**		
Growing season[2]					
2013/2014		53.02^{a}	75.76^{a}	41.43^{a}	43.35^{a}
2014/2015		53.92^{a}	40.01^{b}	37.65^{b}	44.29^{a}
***Azospirillum*[2]**					
Presence		53.04^{a}	59.47^{a}	39.48^{a}	44.21^{a}
Absence		53.90^{a}	56.29^{a}	39.60^{a}	43.43^{a}
Cultivars[3]					
Anta 82 RR®		49.23^{c}	62.75^{a}	38.17^{b}	44.37^{a}
BRS Favorita RR®		50.23^{b}	55.01^{b}	40.69^{a}	44.65^{a}
BRS 820 RR®		49.23^{c}	54.92^{b}	38.76^{b}	40.84^{b}
BRS 780 RR®		59.15^{a}	58.86^{a}	40.53^{a}	45.43^{a}

[1]** and *significant for 1 and 5% of probability, respectively, for F test. ns: Non-significant; MS: means square; DF: degree of freedom; CV: coefficient of variation. [2]Means followed by the same letter have no difference, according to F test. [3]Means followed by the same lowercase in the column are from the same group, according to Scott Knott (1974) test at 5% of probability.

number of grains per pod affected directly grain yield and higher harvest index. On the other hand, the thousand-grain weight was lower for this growing season, presumably by the increase of drain (number of pods and number of grain per pod), with less amount of photoassimilates for each grain.

The efficiency of *A. brasilense* soil inoculation on soy culture can be related to the low competition between the soil microflora and the native bacteria from *Azospirillum* genus (Didonet et al., 2000). The authors also conclude that besides the inoculant quality, the inoculation process is crucial to achieve a higher number of viable bacteria. Therefore, it is possible that a competition with *Bradyrhizobium* or native bacteria prevented the beneficial effect of *A. brasilense* on the development of agronomic traits and the soy grain yield on these study conditions.

Studies that showed benefits in using *A. brasilense* in leguminous crops (Hungria et al., 2013, 2015) did not showed benefits of the leguminous inoculation with *Bradyrhizobium japonicum* and *Azospirillum*, possible because of an increase in the nodulation and N_2 fixation or even due to indirect factors that can be involved. The same authors describe that the resistance to water deficit can be increased and Yadegari et al. (2008) affirm that this is due to the involved bacteria. Otherwise, even with a low rainfall index observed on February and March of 2013/2014 (Figure 1), these benefits were not observed in the present study.

In a general manner, cultivars presented satisfactory

Table 3. Variance analysis and means for plant height at harvest (PHH), insertion of the first pod (IFP), number of pods per plant (NPP), number of grains per pod (NGP), thousand-grain weight (TGW), grain yield (GY) and grain harvest index (GHI) on RR® soy cultivars at maturation (R_8), with inoculation and non-inoculation of *Azospirillum brasilense* with *Bradyrhizobium japonicum* on RR® soy cultivars, to 2013/14 and 2014/15 growing seasons. Lavras-MG, Brazil.

Cause of variation	GL	ANAVA (QM)[1]						
		PHH	IFP	NPP	NGP	TGW	GY	GHI
		cm		unity		g	Kg ha^{-1}	-
Bloco (Bl)	2	26.64	0.54	1.37	0.01	39.44	19828.26	0.0028
Year (Yr)	1	3898.80**	0.27^{ns}	3560.40**	0.66**	722.68**	8037206.39**	0.1435**
Cultivar (C)	3	81.48*	34.20**	137.92*	0.07^{ns}	4049.74**	1218618.91**	0.0080^{ns}
Azospirillum (Az)	1	18.00^{ns}	0.12^{ns}	65.80^{ns}	0.16^{ns}	10.87^{ns}	9157.97^{ns}	0.0002^{ns}
Yr × C	3	38.32^{ns}	15.38**	251.19**	0.27*	720.77**	543960.63**	0.0061^{ns}
Yr × Az	1	7.52^{ns}	0.96^{ns}	11.40^{ns}	0.04^{ns}	196.62^{ns}	35154.75^{ns}	0.0009^{ns}
C × Az	3	19.20^{ns}	3.91^{ns}	97.42^{ns}	0.04^{ns}	10.46^{ns}	200718.36^{ns}	0.0040^{ns}
Yr × C X Az	3	22.28^{ns}	7.53^{ns}	52.88^{ns}	0.21^{ns}	50.54^{ns}	258302.26^{ns}	0.0012^{ns}
Bl × Yr	2	6.95^{ns}	0.68^{ns}	18.31^{ns}	0.04^{ns}	360.52**	63261.64^{ns}	0.0006^{ns}
Error	28	19.75	2.63	53.69	0.08	87.90	144445.51	0.0028
Mean	-	70.77	13.55	59.10	2.07	158.04	3583.63	0.50
CV (%)	-	6.28	11.67	12.40	13.77	5.93	10.61	10.95
Factors		**Means**						
Growing season[2]								
2013/2014		79.78^{a}	13.47^{a}	67.71^{a}	2.19^{a}	154.16^{b}	3993^{a}	0.56^{a}
2014/2015		61.75^{b}	13.62^{a}	50.49^{b}	1.95^{b}	161.92^{a}	3175^{b}	0.45^{b}
***Azospirillum*[2]**								
Presence		71.38^{a}	13.50^{a}	60.27^{a}	2.01^{a}	157.57^{a}	3597^{a}	0.50^{a}
Absence		70.15^{a}	13.60^{a}	57.93^{a}	2.13^{a}	158.52^{a}	3570^{a}	0.50^{a}
Cultivars[3]								
Anta 82 RR®		72.35^{a}	11.13^{b}	57.36^{b}	2.06^{a}	133.77^{c}	3532^{b}	0.50^{a}
BRS Favorita RR®		66.88^{b}	13.65^{a}	55.18^{b}	2.08^{a}	177.99^{a}	3229c	0.53^{a}
BRS 820 RR®		72.15^{a}	14.80^{a}	62.13^{a}	2.16^{a}	163.03^{b}	4003^{a}	0.47^{a}
BRS 780 RR®		71.70^{a}	14.61^{a}	61.73^{a}	1.96^{a}	157.37^{b}	3571^{b}	0.50^{a}

[1]** and *significant for 1 and 5% of probability, respectively, for F test ns: Non-significant; MS: means square; DF: degree of freedom; CV: coefficient of variation. [2]Means followed by the same letter have no difference, according to F test. [3]Means followed by the same lowercase in the column are from the same group, according to Scott Knott (1974) test at 5% of probability.

agronomic characteristics (Table 2). On the economic point of view, the grain yield is more important than the other components, and all cultivars presented values above average for the growing season in Minas Gerais state - 2658 kg ha^{-1}, achieved during 2014/2015 growing season (CONAB, 2015). The BRS 820 RR® resulted in the grain yield of 4003 kg ha^{-1}, 50% more than the grain yield for the 2014/2015 growing season. However, it should be noticed that BRS 820 RR® is the latest cultivar (RM 8.2) when compared with the others.

For the interaction between cultivar × growing season, it was observed that the first pod insertion height for the cultivar Anta 82 RR® was lower, mainly during the 2014/2015 growing season (Figure 2A). For the number of pods per plant, BRS 829 RR® presented the higher mean for the 2013/2014 growing season (Figure 2B). During the 2013/2014 growing season, no statistical differences were observed between cultivars, otherwise, when the subsequent growing season were evaluated, it was observed that BRS 820 RR® presented the higher values (Figure 2C). For thousand-grain weight, BRS Favorita RR® presented the highest mean during the two evaluated years, but during the 2014/2015 crop year it was not different from BRS 820 RR® cultivar (Figure 2D). For the grain yield, the cultivars BRS 820 RR® and BRS 780 RR® presented better performance for the 3013/2014

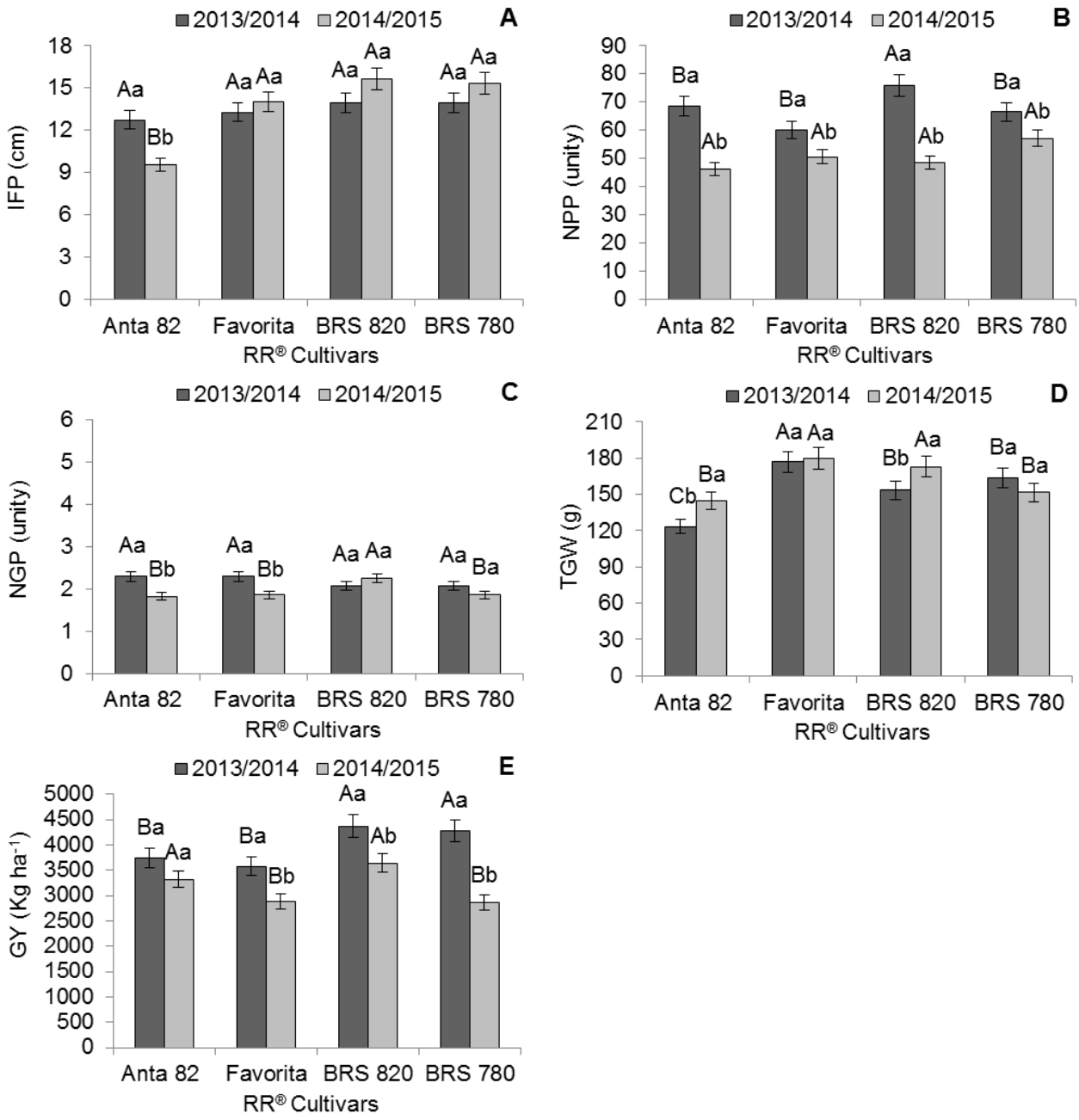

Figure 2. Insertion of the first pod– IFP (A), number of pods per plant – NPP (B), number of grains per pod – NGP (C), thousand-grain weight – TGW (D), grain yield – PG (E) of RR® soy cultivars for the 2013/2014 and 2014/2015 growing seasons, in Lavras, MG, Brazil. Means followed by the same letter, upper case on the same growing season and lower case for the same cultivar, are from the same group, according to Scott Knott (1974) test at 5% of probability.

growing season and the cultivars Anta 82 and BRS 820 RR® during the 2014/2015 growing season, showed the plasticity of the BRS 820 RR® cultivar (Figure 2E).

As previously reported, the interaction between growing season × cultivar is an expected fact and in addition, each cultivar can present intrinsic characteristics and according to the environmental conditions, the agronomic and productive characteristics can be influenced. Thus, characteristics like plasticity of a cultivar are very important, so it can have the ability to modify its morphology depending on environmental conditions.

According to the results and taking into consideration the conditions that this study was conducted, the use or not of *A. brasilense* inoculation, associated with *B. japonicum* did not affect the evaluated agronomic variables and the grain yield in RR® soy cultivars.

Conflict of interests

The authors have not declared any conflict of interests.

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Full Length Research Paper

Microbiota of freshwater catfish species, Filhote (*Brachyplatystoma filamentosum*) and Dourada (*Brachyplatystoma rousseauxii*) from the Amazon Region (Belém-Pará-Brazil)

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This paper identified the bacterial flora composition and its influence on the microbiological spoilage process in economically important fish species: Filhote (*Brachyplatystoma filamentosum*) and Dourada (*Brachyplatystoma rousseauxii*) in the Amazon region. Microbiological characterization was performed: counts of total mesophilic aerobic bacteria, psychrotrophic bacteria and coliforms at 35 and 45°C, respectively. Bacteria were also isolated through seeding in agar surface using Violet Red Bile Glucose (VRBG) for enterobacteria strains and Baird-Parker Agar with Egg-Yolk Tellurite for *Staphylococcus* species, both with incubation at 36°C for 48 h. The bacteria isolated were identified using the API 20E kit (Enterobacteria), and Gram-positive bacteria with API Staph (Staphylococci). Finally, the lag phase for strain growth was measured using spectrophotometry readings (620 nm) at different temperatures (10, 15 and 37°C). The mesophilic aerobic bacteria counts for fresh fish samples ranged from 5.21 to 7.64. The count ranges of psychrotrophic aerobic bacteria found were 5.11 – 6.91 log CFU/g. They also had an average score above 10^3 MPN/g for total coliforms. The most predominant were *Hafnia alvei*, *Pseudomonas luteola*, *Staphylococcus xylosus* and *Staphylococcus lugdunensis.* When subjected to temperature of 10°C, the strains achieved growth after 6 h ($p<0.05$).

Key words: Spoilage, catfish, *Brachyplatystoma filamentosum, Brachyplatystoma rousseauxii.*

INTRODUCTION

Fish is one of the most nutritionally complete foods due to the availability of large amounts of essential nutrients such as high-biological-value protein, vitamins- especially A and D, lipid fraction, high unsaturated fatty acids content and low cholesterol concentration (Córser et al., 2000; Koffi-Nevri et al., 2011). These nutrients present in fish provide a good medium for microbial growth responsible for the overt spoilage and thereby establishes

product shelf life (Oliveira et al., 2008; Gram, 1995; Gram and Huss, 1996).

The spoilage of fresh fish by microbial activity is usually due to its microbiota located mainly in the outer surfaces (skin and gills) and in the intestines of live and newly caught fish (Amaral and Freitas, 2013). It can also be the consequence of fish cross-contamination associated with inappropriate handling and storage (Cruz-Romero et al., 2008). It is known that the flora in tropical fish often carries a slightly higher load of Gram-positive and enteric bacteria than fish from temperate waters. But also, it can be similar to that flora dominated by psychrotrophic Gram-negative, rod-shaped bacteria belonging to the genera Pseudomonas, Moraxella, Acinetobacter, Shewanella, Flavobacterium, Vibrionaceae, Aeroemonadaceae and to a lesser degree, Bacillus, Micrococcus, Clostridium, Lactobacillus and Corynebacterium (Liston, 1980; Apun et al., 1999; Austin, 2002; ICMSF, 2005).

In the Amazon River (Brazil), fish species represent one of the most important resources and significantly contribute to the local economy, playing a vital role in the local diet as one of the primary sources of protein for the majority of the population (Angelini et al., 2006). The catfish including filhote and dourada constitute a valuable resource and are exported to several countries (Angelini et al., 2006).

Filhote or piraiba (*Brachyplatystoma filamentosum*) is one of the most consumed fish species in the Amazon region. It holds great commercial value for its good taste and high yield in the filleting process. It can reach up to 2.50 m in length and 300 kg in weight. It is also considered the largest species of catfish from South America and one of the world's largest (Petrere Junior et al., 2004).

Brachyplatystoma rousseauxii is a large (>1.5 m) migratory catfish of the family Pimelodidae, commonly known as dorado in Bolivia and dourada in Brazil (Carvajal-Vallejos et al., 2014). It is one of the most emblematic species of the Amazon basin, owing to its economic importance and exceptional life cycle, which involves the largest known freshwater migration (Barthem and Goulding, 1997; Alonso, 2002).

The specific microbiota of dourada and filhote are not yet known. The objective of this paper was to identify microbiota and to determine the lag phase of isolated microorganisms to improve the refrigeration temperature conditions in the fish species filhote (B*rachyplatystoma filamentosum*) and dourada (B*rachyplatystoma rousseauxii*) from the Amazon region.

MATERIALS AND METHODS

Fish samples

Successive commercial-sized fish samples (n=4) (Filhote and Dourada) were collected between March and May 2014. At the time of collection, the samples were placed in sterile bags kept under refrigeration (around 10°C) and transported to the Laboratory of Food Microbiology (Federal University of Pará - UFPA) for further analysis.

Microbiological analysis

To analysis, 25 g of each sample (ventral part of the filet) were aseptically collected and added to 225 mL of 0.1% sterile peptone water (SPW), thus obtaining 1:10 dilution, which were homogenized in a stomacher (STOMACHER 400 CIRCULATOR SEWARD) at 2,300 rpm for 30 s. Next, counts of total mesophilic aerobic bacteria, psychrotrophic bacteria and coliform at 35 and 45°C were performed according to Brazil (2003). The total mesophilic aerobic and psychrotrophic bacteria counts were carried out in pour plate using plate count agar followed by incubation at 35°C/48 h for mesophilic and 7°C/10 days for psychrotrophic bacteria. Coliforms at 35 and 45°C were counted through the most probable number (MPN), with three sets of three tubes. Lauryl sulfate tryptose broth (LST) was used as a presumptive medium and incubated at 35°C for 24-48 h. The positive tubes were transferred to brilliant green bile broth 2% (GB) and *Escherichia coli* (EC) broth. The former was incubated at 35°C/24 - 48 h for confirmation of total coliforms and EC broth tubes were incubated in a water bath at 45.5°C/24 h for confirmation of thermo-tolerant coliforms.

Bacteria isolation

The homogenized matter used for microbiological characterization was subsequently used for bacteria isolation. Colonies were isolated from VRBG and Baird-Parker with egg-yolk Tellurite plates after incubation for 48 h at 36°C. Next, one plate was selected for each medium and 5-10 colonies per plate were randomly chosen. The selected colonies were sub-cultured in VRBG or Baird-Parker agar plates. After incubation at 36°C/48 h, one colony was transferred from each plate to brain heart infusion (BHI) with 10% glycerol and stored in a freezer to be used for further tests.

Bacterial strain identification

The bacteria isolated were previously identified with Gram stain tests. Next, Gram-negative strains were identified using the API20E kit (Enterobacteria), and Gram-positive strains with API Staph

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Table 1. Mean values of microbiological characterization in fresh fish.

Samples	Mesophilic aerobic bacteria (log CFU/g)	Psychrotrophic bacteria (log CFU/g)	Total coliforms (MPN/g)	Thermotolerant Coliforms (MPN/g)
Filhote 01	7.49 ± 0.02[a]	6.91 ± 0.02[a]	1.100[a]	1.100[d]
Filhote 02	5.27 ± 0.13[c]	5.69 ± 0.05[b]	1.100[a]	150[b]
Filhote 03	5.21 ± 0.09[c]	5.54 ± 0.02[b]	1.100[a]	1.100[a]
Filhote 04	5.43 ± 0.07[c]	5.11 ± 0.05[d]	1.100[a]	23[e]
Dourada 01	6.02± 0.11[b]	5.53 ± 0.04[b]	1.100[a]	43[a]
Dourada 02	5.18 ± 0.10[c]	5.65 ± 0.03[b]	1.100[a]	210[c]
Dourada 03	5.14 ± 0.09[c]	5.33 ± 0.01[c]	1.100[a]	1.100[a]
Dourada 04	7.64 ± 0.04[a]	5.17 ± 0.08[c, d]	1.100[a]	93f

*Different letters in each column indicate difference at 95% level of significance.

(Staphylococci). The procedure was in accordance with the manufacturer's recommendations (Biomérieux, France) (Harrigan, 1998).

Lag phase determination

Strains were reactivated in nutrient broth for 24 h at 36°C. After that, the isolates identified were transferred to a new nutrient broth (1:15 mL) and maintained at different temperatures: 37, 10 and 15°C during different times: 0, 2, 4, 6, 7, 8 and 9 h (15 and 37°C) and 0, 3, 6, 9 and 10 h (10°C). Spectrophotometric readings were performed in triplicate (Spectrophotometer Model Nova 2000 UV) at 620 nm (Adapted from Damasceno et al., 2015).

Statistical analysis

Tukey's test was applied to evaluate the difference of means among microorganism groups (mesophilic and psychrotrophic bacteria, total and thermotolerant coliforms) found in different fishes. The optical density (lag phase determination) data were subjected to ANOVA considering different groups of isolated microorganisms. The software Statistica 8.0 was applied considering a 95% level of significance.

RESULTS AND DISCUSSION

Microbiological characterization

The mesophilic and psychrotrophic aerobic bacteria count, as well as total and thermotolerant coliform values, showed variation considering 95% significance level. The mesophilic aerobic bacteria counts for fresh fish samples ranged from 5.21 to 7.49 log CFU/g for filhote and 5.14 to 7.64 log CFU/g for dourada (Table 1).

Brazil (2001) does not establish microbiological standards for mesophilic bacteria count in fresh fish. However, the International Commission on Microbiological Specifications for Foods (ICMSF, 1986) recommends the limits for mesophilic aerobic should not exceed values of 10^7 CFU/g or cm^2 in chilled fish samples for human consumption. Considering this limit, most of the samples were within the standards (except sample 1 for Filhote and sample 4 for Dourada).

Previous studies by Shewan (1977), Guizani et al. (2005) and Ercolini et al. (2009) indicated that mesophilic microorganisms are dominant in tropical fish species. Oku and Amakoromo (2013) found total mesophilic values of 10^8 to 10^{10} log CFU/g in *Clarias angularis*, *Channa obscura* and *Chrysicthtys auratus*. Thong et al. (2013) found total mesophilic counts on raw pangasius fish of about 5.1 log CFU/g. Vishwanath et al. (1998) observed a total mesophilic bacteria count ranging from 10^6 to 10^7 CFU/g for *Muscodor albus* (Manipur, India). Damasceno et al. (2015) found mesophilic bacteria values ranging from 4.52 to 8.23 CFU/g in Piramutaba (*Brachyplatystoma vailantii*) and Butterfly peacock bass (*Cichla ocellaris*), respectively, in tropical water. The high count of this microorganism in food may result from unsatisfactory storage conditions, with potential danger to health (Morton, 2001; Coelho et al., 2010; Franco and Landgraf, 2005).

Thus, it appears that mesophilic bacteria are relevant in characterizing the food handling conditions, so it is very important that the current legislation set limits for these microorganisms in fresh fish in order to ensure higher quality (Lopes et al., 2012).

The count of psychrotrophic aerobic bacteria ranged from 5.11 to 6.91 log CFU/g for filhote and from 5.17 to 5.65 log CFU/g for dourada (Table 1). The Brazilian legislation establishes no maximum limit for psychrotrophic microorganisms in fish for human consumption. But considering the ICMSF (1986), filhote and dourada species showed values above the maximum limit (10^7 CFU/g).

In refrigerated fish, the psychrophilic and psychrotrophic bacteria play direct roles in fish deterioration because they

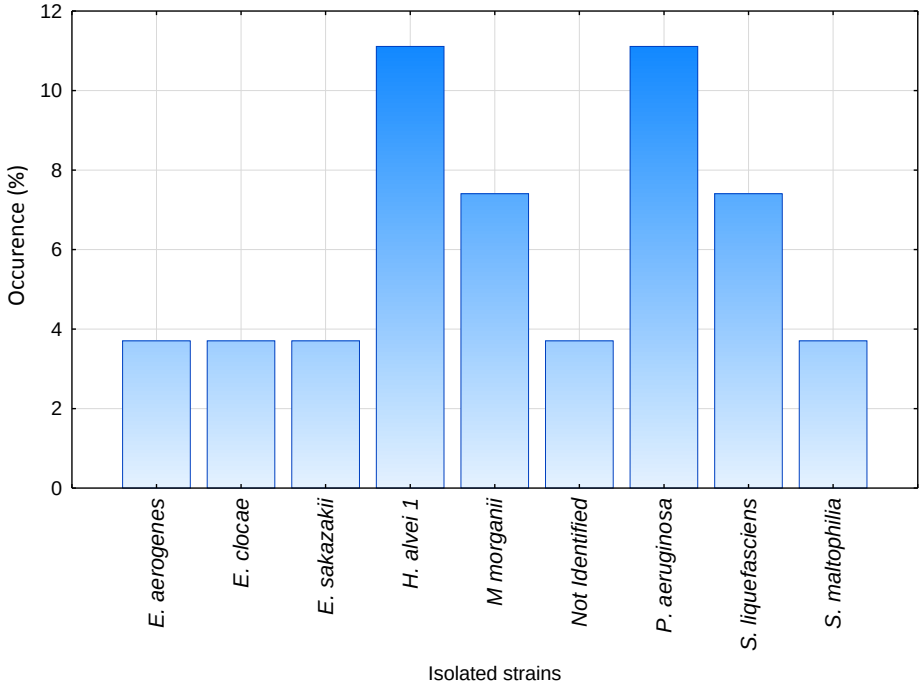

Figure 1. Percentage of occurrence (%) of gram-negative strains isolated from Dourada.

multiply well in these conditions (Franco et al., 1996). Bal'a et al. (2000) found psychrotrophic counts of about 10^3 – 10^7 in fresh fillets of channel catfish (*Ictalurus punctatus*).

Lazarin et al. (2011) found psychrotrophic values of 6.54 log CFU/g in pintado (*P. coruscans*) fillets. Rodrigues et al. (2008), while studying the quality of tilapia (*Oreochromis niloticus*), observed heterotrophic aerobic psychrotrophic microorganism counts between 0 and 7.90 log CFU/g in skinned muscle. Although, the current legislation does not establish limits, the ability of these microorganisms to deteriorate fish through proteolytic processes, even at freezing temperatures, is widely known, which would reduce the product's shelf life (Santos et al., 2008).

The Filhote and Dourada collected had an average score above 1,100 MPN/g for total coliforms (Table 1). Although, this does not indicate the presence of pathogens, total coliforms are important indicators of potential product deterioration and its mean shelf life (Agnese et al., 2001). Brazil (1997) and Brazil (2001) set the value of 10^2 MPN/g as the maximum acceptable standard for thermotolerant coliforms in fish and fishery products. Except for samples 4 for Filhote and 1 and 4 for Dourada, the others showed values that exceeded those established by the Brazilian legislation. Lopes et al. (2012) found total and thermotolerant coliform values ranging from 3.0 to 93 MPN/g in brackish-water grey snapper (*Cynoscion acoupa*). Araújo et al. (2012) observed values of 2,400 MPN/g and 11 to 150 MPN/g for total and fecal coliforms, respectively, in freshwater tambaqui (*Colossoma macropomum*). Oku and Amakoromo (2013) found values of 15 to 43 MNP/g for thermo-tolerant coliforms in the freshwater fish species *C. angularis, C. obscura* and *C. auratus.*

Total and thermo-tolerant coliforms are indicators of hygienic quality, not representing direct contact of the product with human or animal feces, but showing the degree of microbial pollution to which the food has been exposed. This score thus indirectly reflects the quality of production practices.

Bacterial strain identification

Among the 51 isolates, it was observed that 52.94% were Gram negative and the other 47.06% were Gram positive. From the results of the Gram stain test, the kits were selected to identify the strains. The data show that Gram-negative bacteria belonged to 15 different species, described in Figures 1 and 2. The most predominant were *Hafnia alvei* (11.1%) for Dourada and *Pseudomonas*

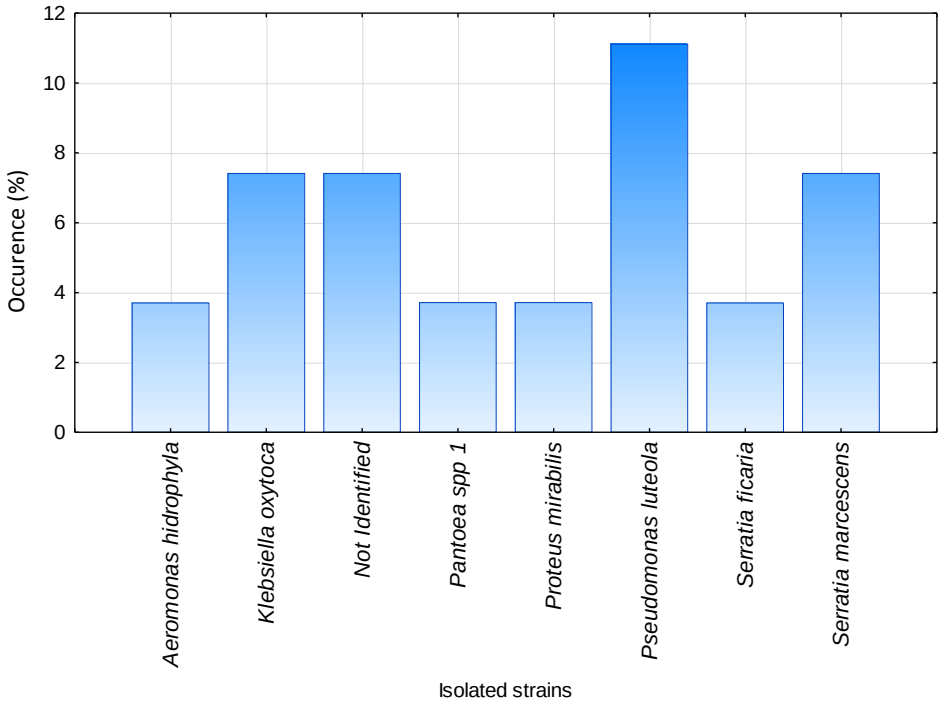

Figure 2. Percentage of occurrence (%) of Gram-negative strains isolated from Filhote.

luteola for Filhote (11.1%). In addition, the incidence of *Serratia marcencens*, *Klebsiella oxytoca*, *Stenotrophomonas maltophilia* and *Aeromonas hidrophyla* was observed, which are opportunistic pathogens but can also cause fish spoilage (Holt et al., 1994).

H. alvei is widely distributed in nature and has been found in a variety of mammals, fish and birds, and also in soil, sewage, freshwater and a number of foods such as meat and dairy products (Lindberg et al., 1998, Vivas et al., 2008). The pathogenicity of *H. alvei* is important in the food industry and infection outbreaks have been reported leading to septicemia in commercial laying hens, pullets and rainbow trout (Janda and Abbott, 2006; Liu et al., 2007; Crandall et al., 2006). In humans, *H. alvei* has been shown to be predominantly associated with several intestinal disorders, including gastroenteritis. Outbreaks or case reports of Hafnia associated with enteric infections have been chiefly reported (Hernandez-Milan and Nenendez-Rivas, 1998; Laguna et al., 1992; Orden and Franco, 1994; Reina et al., 1993; Reina and Borrell, 1991; Seral et al., 2001).

The genus Pseudomonas is known to change many protein-rich foods such as milk, eggs, meat, marine-based foods such as fish and shrimp, and vegetables. According to Franco and Landgraf (2006), pseudomonas are important in food due to their intense metabolic activity, being able to use a wide variety of organic compounds and produce water-soluble pigment and proteolytic and lipolytic enzymes.

P. luteola has been identified as a cause of infection in patients with underlying medical disorders (Otto et al., 2013; Anzai et al., 1997; Kiska and Gilligan, 1999, Connor et al., 1987; Hawkins et al., 1991; Rohav et al., 1995). The normal habitat of *P. luteola* is unclear; it is frequently found in soil, on plants, and in aqueous and damp environments (Freney et al., 1988; Silver et al., 1985; Hawkins et al., 1991). *Pseudomonas aeruginosa* is recognized as belonging to the normal flora of plant surfaces, human skin and animals, and can form biofilms on some surfaces or substrates (Maia et al., 2009). The *P. aeruginosa* species is the most important opportunistic pathogen in humans (Massaguer, 2006; Maia et al., 2009).

The family Enterobacteriaceae has been frequently isolated from the digestive tracts and flesh of freshwater fish (Austin, 2002; Yagoub, 2009; Gonzalez-Rodriguez et al., 2002; Paludan-Müller et al., 1998). Apun et al. (1999) showed some species of Enterobacteriaceae family such as *K. pneumoniae*, *E. aerogenes* and *Escherichia coli* have been isolated from the intestines of tropical freshwater fish. *Serratia* spp. have also been found in

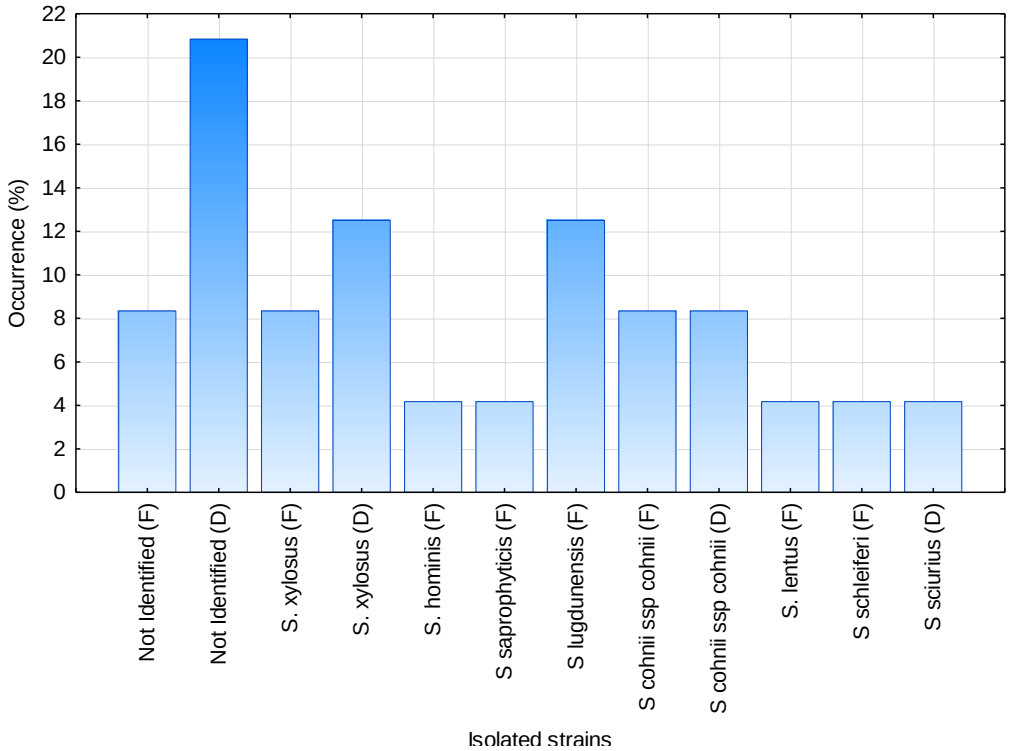

Figure 3. Percentage of occurrence (%) of Gram-positive strains isolated from Filhote (F) and Dourada (D).

Pangasius fillets (Thong et al., 2013). At room temperature (25°C), the microbiota is dominated by mesophilic *Vibrionaceae* (Gorczyca and Len, 1985; Gram et al., 1990) and, particularly if the fish are caught in polluted waters, mesophilic Enterobacteriaceae become dominant (Gram, 1992).

The Gram-positive bacteria found belong to eight different species of staphylococcus as shown in Figure 3. The most prevalent were *Staphylococcus xylosus* (12.50%) and *Staphylococcus lugdunensis* (12.50%) in dourada and filhote, respectively. Damasceno et al. (2015) found predominantly, *S. hominis*, *S. aureus* (Gram-positive), *S. maltophilia* and *E. intermedius* (Gram-negative) in Piramutaba (*Brachyplatystoma vailantii*) and Butterfly peacock bass (*Cichla ocellaris*).

The genus Staphylococcus contains 41 validly described species (DSMZ, 2008) that are traditionally grouped into coagulase-positive (CPS) and coagulase-negative staphylococci (CNS). The CNS *S. xylosus* strains play a significant role in food production. They can be used as starter cultures for the production of fermented meat products such as fermented sausages (Hammes and Hertel, 1998; Chajecka-Wierzchowska et al., 2015). They can also degrade biogenic amines *in vitro* (Martuscelli et al., 2000). *S. xylosus* have been identified in high numbers in dried salted cod (Vilhelmsson et al., 1997; Doe and Heruwati, 1988) and are rarely associated with human or animal infections (Kloos and Schleifer, 1986). *S. lugdunensis* is a common human skin commensal (Bellamy and Barkham, 2002; Vandenesch et al., 1995; Van der Mee-Marquet, 2003). These bacteria display pathogen characteristics, although they do not belong to this group, and exhibit pathogenicity similar to *Staphylococcus aureus*, with high associated morbidity and mortality (Cercenado, 2009; Frank and Patel, 2008; Poutanen and Baron, 2001). Considering that *S. lugdunensis* is not part of the normal fish microbiota, its presence in fish means there have been contamination from human sources.

Lag phase

Among the different 23 strains isolated, 13 bacteria were selected to determine the lag phase under different temperature conditions (Figures 4, 5 and 6). There was a significant variation ($p<0.05$) in optical density of the species studied at temperatures of 37, 15 and 10°C (Tables 2, 3 and 4).

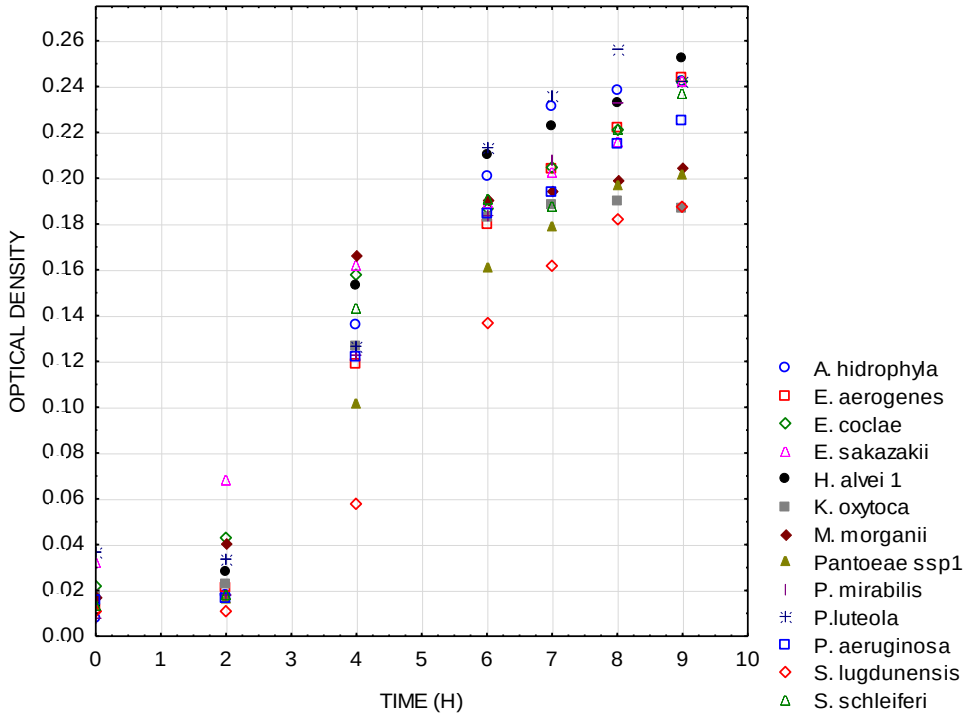

Figure 4. Lag phase measured through spectrophotometry at 37°C.

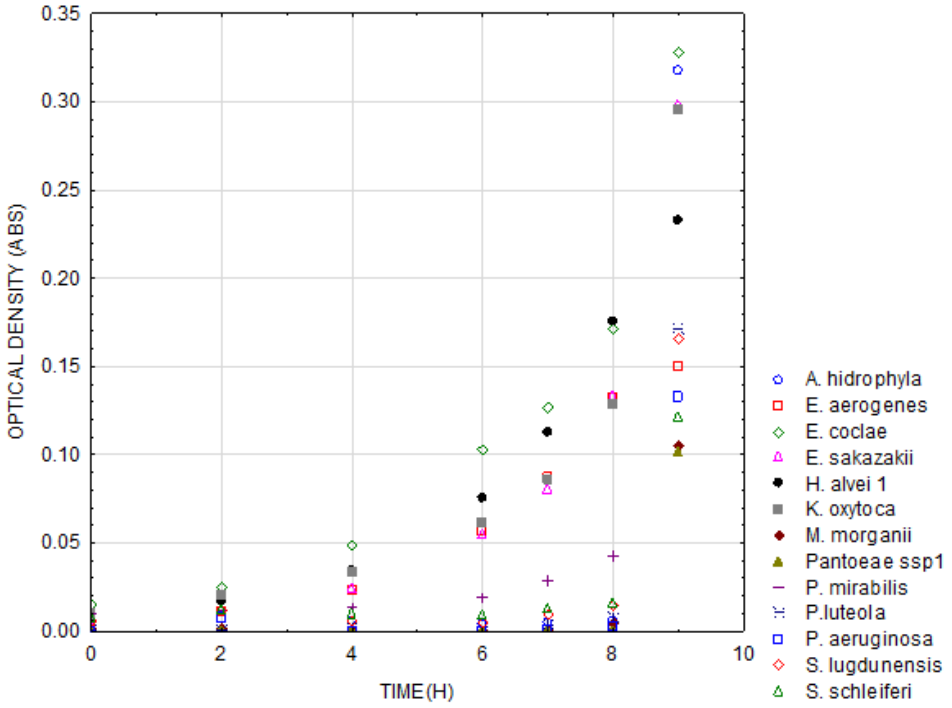

Figure 5. Lag phase measured through spectrophotometry at 15°C.

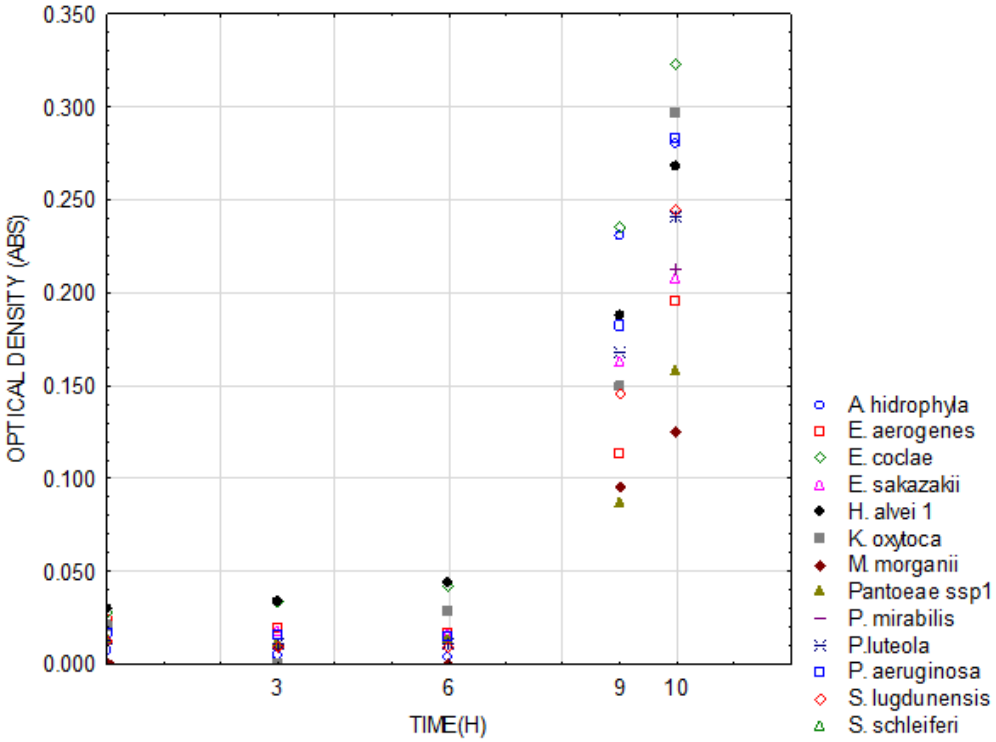

Figure 6. Lag phase measured through spectrophotometry at 10°C.

Table 2. ANOVA for optical density measured through spectrophotometry (620 nm) at 37°C.

Bacterial strains/temperature	F	p
Aeromonas hidrophyla	351.12	0.00
Enterobacter aerogenes	6492.10	0.00
Enterobacter clocae	1495.46	0.00
Enterobacter sakazakii	1229.58	0.00
Hafnia alvei 1	4217.81	0.00
Klebsiella oxytoca	1841.33	0.00
Morganella morganii	3786.19	0.00
Pantoea spp. 1	3858.13	0.00
Proteus mirabilis	815.01	0.00
Pseudomonas luteola	1589.93	0.00
Pseudomonas aeroginosa	896.03	0.00
Staphylococcus lugdunensis	414.56	0.00
Staphylococcus schleiferi	1274.05	0.00

Table 3. ANOVA for optical density measured through spectrophotometry (620 nm) at 15°C.

Bacterial strains/temperature	F	p
Aeromonas hidrophyla	10.78	0.00
Enterobacter aerogenes	72.31	0.00
Enterobacter clocae	26.68	0.00
Enterobacter sakazakii	117.71	0.00
Hafnia alvei 1	1080.73	0.00
Klebsiella oxytoca	312.63	0.00
Morganella morganii	1232.05	0.00
Pantoea spp. 1	62.23	0.00
Proteus mirabilis	696.90	0.00
Pseudomonas luteola	8324.10	0.00
Pseudomonas aeroginosa	9304.56	0.00
Staphylococcus lugdunensis	3722.63	0.00
Staphylococcus schleiferi	2287.08	0.00

The lag phase of the majority of the bacteria studied (except E. *sakazakii*) at 37°C was approximately 2 h (Figure 4). At 15°C, the lag phase was also at least 2 h and at 4 h, *E. clocae,* E. *sakazakii* and *K. oxytoca* were already in log phase. When they were exposed to temperatures of 10°C, the lag phase was at least 6 h.

Table 4. ANOVA for optical density measured through spectrophotometry (620 nm) at 10°C.

Bacterial strains/temperature	F	p
Aeromonas hidrophyla	505.01	0.00
Enterobacter aerogenes	164.45	0.00
Enterobacter clocae	645.99	0.00
Enterobacter sakazakii	88.74	0.00
Hafnia alvei 1	614.83	0.00
Klebsiella oxytoca	32.16	0.00
Morganella morganii	446.89	0.00
Pantoea spp. 1	421.68	0.00
Proteus mirabilis	632.45	0.00
Pseudomonas luteola	1271.25	0.00
Pseudomonas aeroginosa	1184.08	0.00
Staphylococcus lugdunensis	417.88	0.00
Staphylococcus schleiferi	283.53	0.00

This means that a safe condition of refrigeration temperature/time to prevent the multiplication of these microorganisms is about 10°C/6 h. Damasceno et al. (2015) noted that 16 strains isolated from Piramutaba (*Brachyplatystoma vailantii*) and Butterfly peacock bass (*Cichla ocellaris*) did not achieve growth for 6 h at 10°C.

This result is consistent with the FDA (2011), which suggests that raw fish should be kept at 10°C throughout processing to inhibit the growth and toxin production of pathogenic bacteria. Thus, the adoption of correct measures in the fish industry, such as appropriate conservation through cold treatment and maintaining hygienic practices, tends to reduce the risk of transmitting the disease-causing agents and is able to produce a quality product at the end of the production chain (Lopes et al., 2012).

Conclusion

The microbiological assessment of filhote and dourada suggests these Amazon fish species sold in the Ver-o-Peso market have mesophilic and psychrotrophic bacteria counts within the limits set by the Brazilian legislation. The microorganisms selected after isolation underwent a significant variation ($p<0.05$) when they were subjected to different refrigeration temperatures for up to 10 h, which yields the optimal refrigeration temperature/time condition (10°C/6 h) to prevent the multiplication of these microorganisms.

Conflict of interests

The authors have not declared any conflict of interest.

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