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ISOLATION AND IDENTIFICATION OF GOAT MILK-DERIVED *Lactobacillus paracasei* M104 AND *Pediococcus pentosaceus* M103 AND THEIR POTENTIAL USE AS STARTER CULTURE FOR FERMENTATION

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ABSTRACT

The aims of this study were to isolate and identify lactic acid bacteria from the fresh milk of crossbred Peranakan Etawah goats in Yogyakarta, Indonesia and assess their potential utility in dairy fermentation. Fresh milk samples were collected from three different farms and plated into de Man Rogosa and Sharpe (MRS) agar supplemented with 0.5% ox bile. Colonies were purified with a streaking method followed by morphological and biochemical analysis using Gram staining, a catalase test, tests of motility and spore formation and growth at different temperatures. Molecular identification was based on nucleotide sequencing of 16S rRNA genes. Four isolates, M101, M102, M103 and M104, were identified. Certain features of isolates M101 and M102 were homologous with *Lactococcus garvieae*, isolates M103 and M104 showed a degree of homology with *Pediococcus pentosaceus* and *Lactobacillus paracasei* subsp. *paracasei* respectively. Selected isolates were used to ferment milk at 37°C for 10 hours. After 10 h, milk fermented with *Lactobacillus paracasei* M104 had a pH of 4.21±0.07 and acidity of 1.13±0.05. Milk fermented with *Pediococcus pentosaceus* M103 had a pH of 4.34±0.03 with acidity of 1.18±0.05. *Lactococcus garvieae* had limited ability to acidify milk, producing only a slight change in pH over 10 h. There was no significant difference (P>0.05) in viscosity between milk fermented with *Lactobacillus paracasei* M104 and milk fermented with *Pediococcus pentosaceus* M103. Total viable cells were similar between milk fermented with *Lactobacillus paracasei* M104 and milk fermented with *Pediococcus pentosaceus*. On the basis of their capacity to acidify milk, *Pediococcus pentosaceus* M103 and *Lactobacillus paracasei* M104 were selected for further investigation.

Keywords: Goat milk, lactic acid bacteria, molecular identification, fermentation

INTRODUCTION

Goat farming is an attractive enterprise for small-scale farmers in developing countries and those working sub-prime agricultural land (Pirisi *et al.*, 2007) as goats are well-adapted to grazing on poor or marginal land. The capacity for both meat and milk production is another benefit. The main use of goat milk is for cheese-making in small local dairies, particularly in Mediterranean and south-east European countries, although there are also some big cheese factories in Western Europe (Pirisi *et al*., 2007). Goat milk can be used as a substitute for cow milk in cases of allergy to bovine milk (Kongo *et al*.,1996). To date goat milk has been fermented using lactic acid bacteria (LAB) as a starter culture (del Campo *et al*., 2005). In Indonesia, goat milk has been used for cheese production based on LAB as starters obtained from commercial sources. A number of LAB species have been used for milk fermentation, for example *Lactococcus lactis* in cheese production and *Lactobacillus casei* for souring milk. LAB species used in Indonesian dairy productions are usually isolated from gastrointestinal tract (GIT) or from food products; there have been no reports of use of LAB isolated from local goat milk.

LAB are frequently associated with food and feed fermentation (Axelsson, 2004). LAB species are indigenous to food habitats such as plant-derived products and milk environments. LAB are also naturally associated with the animal mucosae, including the mucosae of the small intestine, colon and vagina. The same LAB species are frequently isolated from diverse sources, implying wide distribution and adaptation to a range of environments (Makarova *et al*., 2006). During fermentation LAB produce organic acids, mainly lactic acid and acetic acid, that decrease pH and increase acidity thus causing rapid acidification of fresh milk. This produces fermented milk products with low pH, such as yoghurt, sour milk and other products. Fermenting milk increases the availability of its nutrients. The aim of this study was to isolate and identify LAB from the fresh milk of crossbred Peranakan Etawah (PE) goats, and evaluate their potential for use as starter culture in milk fermentation.

MATERIALS AND METHODS

Fresh milk preparation, bacterial isolation and identification

Fresh goat milk samples were obtained from three different farms in Yogyakarta, Indonesia. Fresh milk was cooled immediately after milking in an ice box and transported to the laboratory for analysis. An 1ml aliquot of fresh goat milk was added to 9 ml of 0.1% (w/v) sterile peptone water to obtain 10^{-1} dilution. After three-fold serial dilution 0.1 ml aliquots were surface plated on de Man Rogosa and Sharpe (MRS) agar (Merck) supplemented with 0.15% (w/v) ox bile then incubated anaerobically at 37°C for 48 hours. White colonies visible on the plate after incubation were subjected to morphological and physiological analysis, including Gram staining, a catalase test, assessments of shape, spore formation, motility and CO_2 and NH_3 production and comparison of growth at 10ºC and 45ºC. These screening tests were used to select colonies with LAB characteristics which were then subjected to molecular identification using 16S rRNA gene amplification.

Amplification of 16S rRNA gene

Amplification of a 518bp fragment of16S rRNA gene was carried out using PCR with primers based on the conserved region of 16S rRNA gene, namely a forward primer *plb16* (5-AGAGTTTGATCCTGGCTCAG-3) and a reverse primer *mlb16* (5-GGCTGCTGGCACGTAGTTAG-3) (Martin *et al*., 2009). Gene amplification was performed using a PCR thermal cycler. The PCR protocol was as follows: denaturation at 95°C for 30s, annealing at 56°C for 30s, elongation at 72°C for 45s (30 cycles) and a final extension at 72°C for 10 min. Amplified bands were resolved by electrophoresis in 1.6% (w/v) agarose gels and visualised using ethidium bromide staining.

DNA sequencing and phylogenetic analysis

The amplified DNA was sequenced using an Applied Biosystem 3730-XL Analyser at 1stBase Sequencing, Kuala Lumpur, Malaysia. The resulting sequences were used to search the National Centre for Biotechnology Information (NCBI) database using the Basic Local Alignment Search Tool (BLAST) algorithm; the isolates were identified on the basis of the sequences which produced the best match (>97%). Molecular Evolutionary Genetics Analysis (MEGA) 6.0 was used to construct a phylogenetic tree of the sequences based on the neighbour-joining algorithm (Saitou & Nei, 1987).

Milk fermentation

Fresh milk was pasteurised at 85°C for 30 min, followed by cooling to 37°C. Pasteurised milk was inoculated with bacterial cultures (2% v/v) and thoroughly stirred. The inoculated milk was incubated at 37°C for 10h; pH and acidity were analysed every 2 hours during the fermentation period. The pH value of the fermented milk was measured with a pH meter (Hanna model, Romania). Titratable acidity was measured as percentage lactic acid by titrating 0.1 N of NaOH using phenolphthalein as indicator (Hadiwiyoto, 1994). The titration method (AOAC Official Method, 1995) was used for lactose analysis.

Apparent viscosity and syneresis

The apparent viscosity of samples was analysed at 29°C using the procedure described by Tuncturk (2009) using a Brookfield digital rheometer model DV III (Brookfield Engineering Laboratories Inc., Massachusetts, USA) with spindle numbers 62 and 63, spindle speed 60 rpm. Syneresis was measured using centrifugation (Keogh and O'Kennedy, 1998). A 15 g aliquot of sample was centrifuged at 1500 rpm for 20 min at 4°C. The supernatant was collected and weighed and the extent of syneresis was calculated using following equation:

$$\text{Syneresis (\%)} = \frac{\text{Weight of supernatant (g)}}{\text{Weight of sample (g)}} \times 100\%$$

Total viable cells

An 1 ml aliquot of fermented milk was collected from all samples. Aliquots were diluted seven-fold in a sterile solution of 0.85% (w/v) NaCl, then plated onto MRS agar and incubated at 37°C for 24 to 48 h. The colonies were then counted and total bacterial numbers per sample were estimated.

Lactic acid concentration using High Performance Liquid Chromatography (HPLC)

Lactic acid measured using HPLC was carried out according to the procedure described by Bevilacqua and Califano (1989) using a reverse-phase C8 column at room temperature, a mobile phase of 0.5% w/v buffer ($(NH_4)_2HPO_4$ at pH 2.24 with H_3PO_4)-0.4% (v/v) acetonitrile and 1.2 mL/min flow rate with UV detection at 214 nm.

Data analysis

Data on pH, acidity, lactose content, lactic acid concentration, viscosity and syneresis were analysed statistically using paired *T*-tests with statistical significance accepted at $P<0.05$.

RESULTS AND DISCUSSION

Bacterial Identification and Selection

We obtained 4 isolates with the morphological and physiological characteristics of LAB (Table 1). LAB are usually characterised as Gram-positive, aerobic or facultative anaerobic, non-motile asporogenous rods and cocci with the ability to ferment carbohydrate with lactic acid as the main fermentation product (Franz *et al*., 2010). Preliminary identification was based on morphological and physiological analysis, ability to produce CO_2, growth at different temperatures (10°C, 37°C and 45°C), pHs (4.4 and 9.6) and in media containing 6.5% NaCl. The characteristics of all isolates are presented in Table 1.

Table 1 Morphological and biochemical identification of isolates

Isolates	Form	CO_2	Growth temperature			NaCl 6.5%	Growth pH		Genus*
			10°C	37°C	45°C		4.4	9.6	
M101	Cocci	-	-	+	+	-	+	-	*Pediococcus*
M102	Cocci	-	-	+	+	+	-	-	*Lactococcus*
M103	Cocci	-	-	+	+	-	+	-	*Pediococcus*
M104	Bacilli	-	-	+	+	+	+	-	*Lactobacillus*

* Based on Axelsson's (2002) classification of LAB.

Comparison of the fermentation patterns of all isolates with Axelsson's (2002) classification of LAB resulted in identification of three genera of LAB; M101 and M103 were identified as *Pediococci,* M102 as *Lactococcus* and M104 as *Lactobacillus* (Table 1). All isolates were homofermentative without CO_2 production and isolates M101 and M103 were intolerant of salt (NaCl 6.5%). 16S rRNA gene sequencing suggested that isolates M101 and M102 were close homologues (97% similarity) of *Lactococcus garvieae* strain 29; isolate M103 was a close homologue (96% similarity) of *Pediococcus pentosaceus* strain LAB6 and isolate M104 was a close homologue (97% similarity) of *Lactobacillus paracasei* subsp. *paracasei* strain X212 (Figure 1).

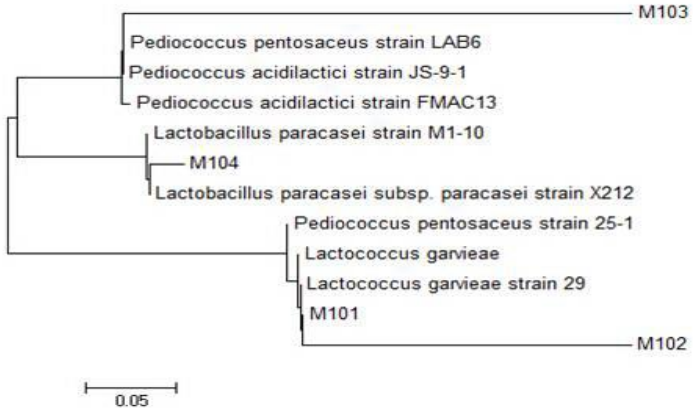

Figure 1 Phylogenetic tree showing the genetic relationship of selected isolates to other LAB

This study identified *L. garvieae, P. pentosaceus* and *L. paracasei* in fresh milk from crossbred Peranakan Etawah goats in Indonesia. *L. garvieae* is often associated with fermented dairy products; Morea, Baruzzi and Cocconcelli (1999) have previously reported its presence in goat milk. *L. garvieae* is one of main bacterial populations in traditional production of mozzarella-type cheese from goat milk and forms part of the autochthonous bacterial population in various Italian and Greek cheeses (Fortina *et al*., 2007). *L. garvieae* has also been reported to occur in the milk of Kabyle goats from Algeria (Badis *et al.*, 2004). This study reports the presence of *L. garvieae* in fresh milk from crossbred Peranakan Etawah goats.

P. pentosaceus is part of the natural microflora of vegetables and dairy products (Carraro *et al*., 2011) and has also been found in human breast milk (Osmanagaogl *et al*., 2013). *P. Pentosaceus* appears to be a good candidate as starter culture for dairy fermentation as it grows well at 45°C, is homofermentative and has a long history as being safe for consumption. In other reports, *P. pentosaceus* isolated from traditional dairy products was good at curdling milk.

L. paracasei has previously been isolated from goat milk (Badis *et al.*, 2004). This species has been used as starter culture for probiotic fermented milk (Kristo *et al*., 2003; Patrignani *et al*., 2009). It inhibits the activity of yeasts on the surface of dairy products such as yoghurt or cheese at refrigerator temperatures (6°C) without an influencing their quality (Schwenninger and Meile, 2004).

Acidification Capability

The acidifying capacity of the selected isolates was evaluated by using them to inoculate individual samples of sterile skimmed milk (18% w/v) and incubating at 37°C for 10 h. *P. pentosaceus* M103 and *L. paracasei* M104 exhibited fast acidification activity (Figures 2 and 3), whereas *L. garvieae* M101 and M102 exhibited slow acidification activity (data not shown). The pH and acidity of milk samples inoculated with *L. paracasei* M104 and *P. pentosaceus* M103 during fermentation are presented in Figures 2 and 3, respectively. At the final pH measurement, isolates *L. garvieae* M101 and M102 had pHs of 5.18 ± 0.12 and 5.24 ± 0.17 respectively, whilst fermentation using *P. pentosaceus* M103 and *L. paracasei* M104 resulted in samples with pHs of 4.34 ± 0.03 and 4.21 ± 0.07, respectively (Figure 2).

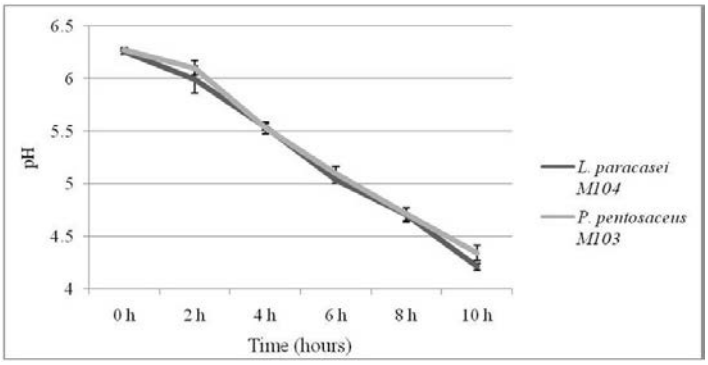

Figure 2 Changes in pH during incubation with different starter cultures

Figure 2 showed that during fermentation pH decreased gradually whilst acidity increased significantly ($P<0.05$) (Figure 3). The acidity of milk fermented with *L. paracasei* M104 increased from 0.31% ± 0.01 to 1.13 ± 0.05 and the acidity of milk fermented with *P. pentosaceus* M103 increased from 0.31% ± 0.01 to 1.18 ± 0.05 (Figure 3); milk fermented with *L. paracasei* M104 or *P. pentosaceus* M103 had similar acidity and pH values.

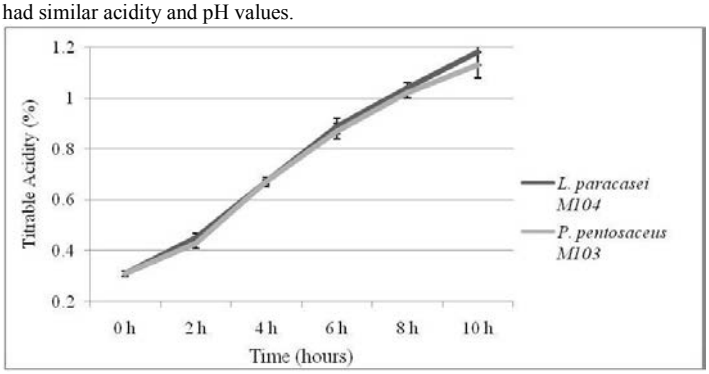

Figure 3 Changes in acidity during incubation with different starter cultures

According to Robinson (2002), acidification is the main indicator of the progress of fermentation. LAB produce organic acids from lactose enzymatically, resulting in formation of curds with distinctive flavours. In this study lactose content and lactic acid concentration were measured before and after fermentation (Table 2). During fermentation the lactose content of milk decreased from 4.6%±0.39 to 3.15%±0.35 when fermented with *L. paracasei* M104, and from 4.6% ± 0.39 to 3.2% ± 0.22 when fermented with *P. pentosaceus* M103 (Table 2), whilst the lactic acid concentration increased from 0.1422% ± 0.021 to 0.1905% ± 0.0145 and from 0.1422% ± 0.021 to 0.1768% ± 0.034 during fermentation with *L. Paracasei* M104 and *P. pentosaceus* M103 respectively (Table 2).

Table 2 Changes in lactose content (%) and lactic acid (%) after 10h fermentation

Starter Culture	Time (hour)	Lactose (%)	Lactic Acid (%)
L. paracasei M104	0	4.60 ± 0.39^a	0.1422 ± 0.0210^b
	10	3.15 ± 0.35^b	0.1905 ± 0.0145^a
P. pentosaceus M103	0	4.60 ± 0.39^a	0.1422 ± 0.0210^b
	10	3.24 ± 0.22^b	0.1768 ± 0.034^a

Values expressed as mean ± standard deviation of three replicates
Means within each column with different superscript letters indicate statistically significant differences (P<0.05)

The increase in acidity during fermentation resulted in coagulation of proteins and curd formation, leading to increased viscosity and syneresis. We therefore measured apparent viscosity and syneresis after fermentation; these data are presented in Table 3. Before fermentation milk had a viscosity of 1.82cP ± 0.12 (Table 3). Milk fermented with *P. pentosaceus* M103 had significantly ($P<0.05$) higher apparent viscosity than milk fermented with *L. paracasei* M104 (1475cP ± 15 and 1075cP ± 25 respectively, at the end of the fermentation period) (Table 3). There were no significant differences (P>0.05) in syneresis between milk fermented with *L. paracasei* M104 or *P. pentosaceus* M103 (84.94% ± 1.6 and 84.94% ± 2.2 respectively) (Table 3).

Table 3 Viscosity (cP) and syneresis (%) before and after fermentation

Starter culture	Viscosity		Syneresis
	0 h	10 h	(10 h)
L. paracasei M104	1.82±0.12^a	1075±25^b	84.94±1.6
P. pentosaceus M103	1.82±0.12^a	1475±15^c	84.94±2.2

Values expressed as mean ± standard deviation of three replicates
Means within each column with different superscript letters indicate statistically significant differences (P<0.05)

Starter cultures can be defined as a microbial biomass, consisting of a large number of cells in the logarithmic phase of growth, which can be added to raw food products to accelerate fermentation processes (Leroy and De Vuyst, 2004). *P. pentosaceus* M103 and *L. paracasei* M104 (Figures 2 and 3) showed fast acidification activity, whereas *L. garvieae* M101 and M102 showed slow acidification activity (data not shown). The primary function of LAB in industrial dairy fermentation is use of lactose (de Vos and Vaughan, 1994). Lactic acid accumulation decreases pH and improves the acidity of products. Fermented dairy products usually have a pH of 4.6 or lower (Chandan, 2006). In this study products fermented with *L. garvieae* M101 and M102 had pHs of no 4.34 ± 0.03 and 4.21 ± 0.07, respectively (Figure 2), i.e. within the required range. A previous study (Widodo *et al.*, 2014) reported that pH 4.5 was achieved after 5 hours of goat milk fermentation using mono-species starter cultures of *Lactobacillus acidophilus* FNCC-0029 or *Lactobacillus casei* FNCC-0051. In this study a pH of 4.5 was obtained after 9 hours of fermentation with *P. pentosaceus* M103 or *L. paracasei* M104 as starter, indicating that fermentation with these species was slower than that of *Lactobacillus acidophilus* FNCC-0029 or *Lactobacillus casei* FNCC-0051. The final acidities of milk fermented with *L. paracasei* M104 or *P. pentosaceus* M103 were 1.13% ± 0.05 and 1.18% ± 0.05 respectively (Figure 3), within the range of the Indonesian national standard (SNI), 0.5 to 2.0% (Badan Standarisasi Nasional, 1998).

Higher acidity stimulates syneresis, thereby improving the viscosity of fermented dairy products (Tamime and Robinson, 1999). In this study use of *P. pentosaceus* M103 or *L. paracasei* M104 for milk fermentation resulted in products with the same final acidity (Table 2), but different viscosities (Table 3). Keogh and O'Kennedy (1998) suggested that differences in viscosity are probably due to variations in protein ratios or ion values, and may also be affected by incubation and storage conditions.

Total Viable Cells

Data on the numbers of viable *L. paracasei* M104 and *P. pentosaceus* M103 before and after fermentation are presented in Table 4. At the end of fermentation there was no significant difference (P>0.05) in the number of viable cells in samples fermented with *P. Pentosaceus* M103 or *L. Paracasei* M104 (Table 4). After fermentation the total number of viable *L. paracasei* M104 cells was 9.23 $\log_{10}$ CFU/ml, an increase of 1.4 $\log_{10}$ CFU/ml, whereas the total number of viable *P. pentosaceus* M13 cells was 9.63 $\log_{10}$ CFU/ml, an increase of 1.9 $\log_{10}$ CFU/ml (Table 4).

Table 4 Viable cells ($\log_{10}$cfu/ml) in milk before and after fermentation

Starter culture	Viable count (CFU/ml)	
(2%, w/v)	0 h	10 h
L. paracasei M104	7.817±0.053^a	9.238±0.330^b
P. pentosaceus M13	7.719±0.159^a	9.637±0.510^b

Values expressed as mean ± standard deviation of three replicates
Means within each column with different superscript letters indicate statistically significant differences (P<0.05)

The total numbers of viable *P. pentosaceus* M103 and *L. paracasei* M104 after fermentation were 9.637 ± 0.510 and 9.238 ± 0.330 $\log_{10}$ CFU/ml respectively; the minimum value required for functional activity in fermented milk products is 10^6 CFU/ml (Shah, 2000). We did not evaluate the probiotic capability of *P. pentosaceus* M103 or *L. paracasei* M104; however several other studies have classified both species as probiotic (Kristo *et al*., 2003; Osmanagaoglu *et al*., 2013; Patrignani *et al*., 2009).

CONCLUSION

This study identified four species of LAB in the milk of Peranakan Etawah goats. Isolates identified as *Lactobacillus paracasei* M104 and *Pediococcus pentosaceus* M103 were shown to acidify skimmed milk effectively and had potential uses as starter culture for milk fermentation.

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ANTIMICROBIAL AND PHYTOCHEMICAL ATTRIBUTES OF *DENNETTIA TRIPETALA* F. BAKER ROOT AND BARK EXTRACTS

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ABSTRACT

The antimicrobial activities and phytochemical constituents of aqueous and hexane extracts of both the bark and roots of *Dennettia tripetala* F. Baker (*Annonaceae*) were determined using routine methods. Flavonoids, saponins, phenolic compounds, volatile oil, carbohydrate and reducing sugars were present in the aqueous extract of the root and bark, but absent in the hexane extracts. The antimicrobial activities of the extracts were tested against bacterial and fungal isolates using agar diffusion method.The commercial antibiotics used as positive reference standards to determine the sensitivity of the isolates were Gentamicin and Fluconazole.The aqueous extract of the root showed inhibitory activity against *Escherichia coli, Bacillus subtilis, Staphylococcus aureus, Enterococcus faecalis* and *Pseudomonas aeruginosa,* with zones of inhibition ranging from 0.01mm to 30mm. The aqueous root extract inhibited the growth of *Pseudomonas aeruginosa* and *Enterococcus faecalis* with MIC values at 50 mg/ml.The results showed that the aqueous root extract possesed antimicrobial attributes as indicated by its activity on test bactetrial isolates. Phytochemicals were not present in the hexane extracts and the extracts showed no activity against the test organisms.

Keywords: *Dennettia tripetala*, aqueous extract, hexane extract, bark, root, phytochemistry and antimicrobial

INTRODUCTION

Records of indigenous knowledge from various parts of the world illustrate an age long tradition of plants being a major bioresource base for health care (**Idu *et al*., 2000a**). It has been found that some drugs are synthesized from plants (**Idu *et al*., 2007**). A medicinal plant can be defined as any plant which in one or more of its organs contain substances that can be used for therapeutic purpose or which are precursors for the synthesis of useful drugs (**Sofowora, 1982**). Plant secondary metabolites constitute one of the most numerous and widely distributed groups of substances in the plant kingdom and have been known to be responsible for the therapeutic activities of medicinal plants **(Ataman *et al.*, 2002).** Phytomedicines derived from plants have shown great promises in the treatment of intractable infections **(Iwu *et* al., 1999)** and *in vitro* screening methods could provide the needed preliminary observations necessary to select crude plant extracts with potentially useful properties for further chemical and pharmacological investigations **(Ozumba, 2003; Pessini *et al.*, 2003)**. *Dennettia tripetala* Baker F. (pepper fruit) belongs to the family Annonaceae. It is found in the tropical rainforest region of Nigeria and occassionally in the savanna region **(Okwu *et al*., 2005)**. The plant flourishes at the onset of the rain, from April through June **(Umoh, 1998).** It is a woody plant of about 7 m in height with simple leaves. The young leaves are chewed on account of their pungent spicy taste. The fruits are initially green then turn red on ripening between April and May and have a peppery spicy taste and are chewed for this property. The leaves are used to treat mild fever with other herbs such as the leaves of mango **(Gill, 1992).** The young stems of this plant are also used as chewing stick by patients with fever to help improve their appetite. Pharmacologically, the oil extracted from the fruits of the plant when mixed with fresh leaves of mango is used in treating fever **(Gill, 1992).**

The aim and objective of this study was to determine the antimicrobial activity and the phytochemical consitiuents of hexane and aqueous extracts of bark and roots of *D. tripetala.*

MATERIAL AND METHODS

Plant materials

The barks and roots of *D. tripetala* were collected in the month of August, 2010 from Umudioka in Dunukofia L.G.A. of Anambra State and was identified by Professor M. Idu of the Department of Plant Biology and Biotechnology, University of Benin, Benin City. The plant parts were dried at an average temperature of 45^{O}C for 3 days using a 50L GALLENKAMP hot box oven (model; N9615-50, 250^{o}C) manufactured by Rigal Bennett. The dried samples were macerated to fine particles. The weighed powdered samples (550g of bark and 165g of root) were extracted with 2100 ml hexane for bark and 700 ml for root with occasional stirring for 48 hrs for maximum dissolution after which it was sieved through a Whatman filter paper to obtain a solution. The solution was concentrated to dryness using 10L GALLENKAMP water bath(model; S8689-50) manufactured by Rigal Bennett. The residue were air-dried to remove any remaining hexane before they were subjected to another extraction using distilled water. The extraction was done for 18 h using 2800 ml of boiled distilled water. They were filtered and then concentrated over Gallenkamp water bath. The four extracts were stored in refrigerator until required for use.

Standardization and preparation of the microbial innocula

The stock culture were obtained from the Department of Pharmaceutical Microbiology, Faculty of Pharmacy, University of Benin. Five (5) bacterial strains; *Bacillus subtilis, Pseudomonas aeruginosa, Escherichia coli, Staphylococcus aureus* and *Enterococcus faecalis* and two (2) fungi strains; *Candida albicans and Microsporium audouinii* were used.

All the test organisms (bacterial and fungal isolates) were sub-cultured on freshly prepared nutrient agar plates and potato dextrose agar plates and incubated for 24h and 48h respectively. Following the slightly modified method of Vandepitte *et al*. (2003), the inocula were standardized by transfering parts of the streaked colonies into 5ml of sterile nutrient broth in test tubes and incubated for 3h at 37^{O}C. The bacteria and fungi suspension growth were appropriately compared to that of a freshly prepared barium sulphate solution(0.5ml of 1% barium in Chloride to 99.5ml of 1% H_2SO_4 (0.36 Normal). The obtained turbidity was adjusted by adding more sterile nutrient broth to match the 0.5 Mcfarland

standard (10^6cfu/ml). After incubation, 1ml of the standardized cultures of the microbial isolates were inoculated onto the surface of freshly prepared nutrient agar plates (for the bacterial isolates) and sabouraud dextrose agar plates (for the fungal isolates) with the aid of sterile bent glass rod.

Test for antimicrobial activity

The diluted aqueous and hexane extracts of the root and bark of the plant were tested for their antimicrobial properties using the punch hole method **(Stoke, 1975).** The concentrations used were 400, 200, 100 and 50 mg/ml. The antibiotics; gentamicin (at a concentration of 5 mg/ml) and commercial fluconazole (at a concentration of 5 mg/ml), were used as positive controls for bacterial and fungal isolates respectively. The standard antiobiotic sensitivity discs (Gentamicin and Fluconazole) made by Asodisks Atlas Diagnostics, Enugu, Nigeria were purchased from a chemical laboratory store in Benin city and used, after which the plates were incubated overnight at $37^OC \pm 2^OC$ and $28^OC \pm 2^OC$ for bacterial and fungal cultures respectively. At the end of the incubation period, the diameter of the inhibition zone(s) were measured using meter rule and recorded.

Minimum Inhibitory Concentration (MIC) Determination

The minimum inhibitory concentration (MIC) of the crude extracts were determined by adapting the punch hole method as described by **Stokes (1975)** and decreased **c**oncentrations of the extracts i.e. 200, 100, 50, 25 and 12.5 mg/ml were utilized. The least concentration of the extract which inhibited the growth of the inocula was considered as the minimum inhibitory concentration.

Phytochemical screening of the extracts of the bark and root

Phytochemical screening of the aqueous and hexane extracts of the bark and root were conducted according to standard procedures as described by **Trease and Evans (1996).** The respective extracts were analysed for the presence of alkaloids, saponins, tannins, volatile oils, anthracene, flavonoids, reducing sugars, carbohydrates, cyanogenetic glycerides, phenolic compounds.

Data analysis

Results were expressed as means ± standard error of means [S.E.M] and level of significance between means were computed by student's t-test using SPSS 14.00 computer software package. The level of significance was determined at 0.05.

RESULTS

The test microbial isolates showed varying degrees of response towards various concentrations of *Dennettia tripetala* aqueous extract of the bark. The maximal or highest inhibitory zone (0.09 mm±0.01) was shown by *P. aeruginosa* exposed to 400 mg/ ml concentration of aqueous bark extract (Tab. 1). *P. aeruginosa* , *E. faecalis* and *E. coli* elicited the least zone of inhibition (0.01 mm±0.00) against 50 mg/ml and 100 mg/ml aqueous bark extract concentrations (Tab.1). The observed differences in the mean inhibitory zones was significant with the zone elicited by *P. aeruginosa* and *E. coli* being responsible**.**

Table 1 The effect of aqueous extract of the bark of *D. tripetala* on the test organisms at various concentrations

Test organisms	Zones of inhibition (mm)			
	400mg/ml	200mg/ml	100mg/ml	50mg/ml
Bacillus subtilis	$0.03^a \pm 0.02$	$0.03^a \pm 0.01$	NMZI	NMZI
Pseudomonas aeruginosa	$0.09^a \pm 0.01$	$0.05^a \pm 0.01$	$0.02^b \pm 0.01$	$0.01^b \pm 0.00$
Escherichia coli	$0.02^a \pm 0.01$	NMZI	$0.01^a \pm 0.00$	NMZI
Staphylococcus aureus	$0.05^a \pm 0.01$	$0.04^a \pm 0.00$	$0.04^a \pm 0.01$	NMZI
Enterococcus feacalis	$0.05^a \pm 0.01$	$0.03^b \pm 0.00$	$0.02^b \pm 0.03$	$0.01^b \pm 0.00$
Candida albicans	NMZI	NMZI	NMZI	NMZI
Microsporium audouinii	NMZI	NMZI	NMZI	NMZI

Legends: Values are means ± S.E.M of two measurements across each zone of inhibition. Means ± S.E.M with different superscript within a row are significantly different (P < 0.05). NMZI-No Measurable Zone of Inhibition.

The maximal or highest inhibitory zone (0.05 mm±0.01) was shown by *P. aeruginosa* exposed to 400 mg/ ml concentration of hexane bark extract (Tab. 2). *B. subtilis* and *S. aureus* elicited the least zone of inhibition (0.02 mm±0.01) against 200 mg/ml and 400 mg/ml hexane bark extract concentrations (Tab.2). The observed differences in the mean inhibitory zones was not significant with the zone elicited by *P. aeruginosa* and *B. subtilis* being responsible.

Table 2 The effect of hexane extract of the bark of *D. tripetala* on the test organisms at various concentrations.

Test organisms	Zones of inhibition (mm)			
	400mg/ml	200mg/ml	100mg/ml	50mg/ml
Bacillus subtilis	$0.04^a \pm 0.01$	$0.02^a \pm 0.01$	NMZI	NMZI
Pseudomonas aeruginosa	$0.05^a \pm 0.01$	$0.03^a \pm 0.01$	NMZI	NMZI
Escherichia coli	$0.05^a \pm 0.01$	NMZI	NMZI	NMZI
Staphylococcus aureus	$0.02^a \pm 0.01$	NMZI	NMZI	NMZI
Enterococcus feacalis	$0.03^a \pm 0.01$	NMZI	NMZI	NMZI
Candida albicans	NMZI	NMZI	NMZI	NMZI
Microsporium audouinii	NMZI	NMZI	NMZI	NMZI

Legends: Values are means ± S.E.M of two measurements across each zone of inhibition. Means ± S.E.M with different superscript within a row are significantly different (P < 0.05). NMZI-No Measurable Zone of Inhibition.

The maximal or highest inhibitory zone (30.00 mm±3.00) was shown by *P. aeruginosa* exposed to 400 mg/ ml concentration of aqueous root extract (Tab. 3). *S. aureus* and *E. coli* elicited the least zone of inhibition (0.02 mm±0.01) against 50 mg/ml and 100 mg/ml aqueous root extract concentrations (Tab.3). The observed differences in the mean inhibitory zones was significant with the zone elicited by *E. coli, S. aureus, E. faecalis* and *B. subtilis* being responsible**.**

Table 3 The effect of aqueous extract of the root of *D. tripetala* on the test organisms at various concentrations.

Test organisms	Zones of inhibition (mm)			
	400mg/ml	200mg/ml	100mg/ml	50mg/ml
Bacillus subtilis	$25.00^a \pm 1.00$	$23.00^a \pm 1.00$	$0.09^b \pm 0.01$	$0.04^b \pm 0.01$
Pseudomonas aeruginosa	$30.00^a \pm 3.00$	$27.00^a \pm 2.00$	$25.00^a \pm 0.50$	$20.00^a \pm 0.75$
Escherichia coli	$13.00^a \pm 1.55$	$10.00^a \pm 0.35$	$0.02^b \pm 0.01$	NMZI
Staphylococcus aureus	$22.00^a \pm 0.10$	$18.00^a \pm 0.20$	$16.00^a \pm 1.56$	$0.02^b \pm 0.01$
Enterococcus feacalis	$20.00^a \pm 1.00$	$17.00^a \pm 0.20$	$16.00^a \pm 0.78$	$15.00^a \pm 050$
Candida albicans	NMZI	NMZI	NMZI	NMZI
Microsporium audouinii	NMZI	NMZI	NMZI	NMZI

Legends: Values are means ± S.E.M of two measurements across each zone of inhibition. Means ± S.E.M with different superscript within a row are significantly different (P < 0.05). NMZI-No Measurable Zone of Inhibition.

The maximal or highest inhibitory zone (0.08 mm±0.01) was shown by *P. aeruginosa* exposed to 400 mg/ ml concentration of hexane root extract (Tab. 4). *B.subtilis*and *E. faecalis* elicited the least zone of inhibition (0.02 mm±0.01) against 100 mg/ml and 200 mg/ml hexane root extract concentrations (Tab.4). The observed differences in the mean inhibitory zones was significant with the zone elicited by *B. subtilis* being responsible**.**

The highest inhibitory zone (0.08mm) was shown by *P. aeruginosa* at 400mg/ml whilst the least inhibition zone (0.02mm) was displayed by*B.subtilis* and *E. faecalis* at100mg/ml and 200mg/ml respectively. The differences in the mean inhibitory zones shown by *B. subtilis* was not significant (P>0.05) (Tab. 4).

Table 4 The effect of hexane extract of the root of *D. tripetala* on the test organisms at various concentrations.

Test organisms	Zones of inhibition (mm)			
	400mg/ml	200mg/ml	100mg/ml	50mg/ml
Bacillus subtilis	$0.05^a \pm 0.01$	$0.02^a \pm 0.01$	$0.02^a \pm 0.01$	NMZI
Pseudomonas aeruginosa	$0.08^a \pm 0.01$	$0.05^b \pm 0.01$	NMZI	NMZI
Escherichia coli	$0.05^a \pm 0.01$	$0.04^a \pm 0.01$	NMZI	NMZI
Staphylococcus aureus	$0.03^a \pm 0.01$	NMZI	NMZI	NMZI
Enterococcus feacalis	$0.04^a \pm 0.01$	$0.02^a \pm 0.01$	NMZI	NMZI
Candida albicans	NMZI	NMZI	NMZI	NMZI
Microsporium audouinii	NMZI	NMZI	NMZI	NMZI

Legends: Values are means ± S.E.M of two measurements across each zone of inhibition. Means ± S.E.M with different superscript within a row are significantly different (P < 0.05). NMZI-No Measurable Zone of Inhibition.

Gentamicin exhibited inhibitory activity against all the exposed bacterial cultures except *B. subtilis.* The highest inhibition zone (50 mm) against gentamicin disc was shown by *S. aureus*. Fluconazole did not show any significance inhibition on the fungal test organisms (Tab. 5).

Table 5 Effects of the antibiotics on the test organisms

Test organisms	Zone of inhibition (mm)	
	Gentamicin (5mg/ml)	Fluconazole (5mg/ml)
Bacillus subtilis	NMZI	NA
Pseudomonas aeruginosa	13	NA
Escherichia coli	29	NA
Staphylococcus aureus	50	NA
Enterococcus feacalis	20	NA
Candida albicans	NA	NMZI
Microsporium audouinii	NA	NMZI

NMZI- No Measurable Zone of Inhibition; NA- Not Applicable

The hexane extract of root and bark gave a negative result for all the secondary metabolites whereas the aqueous extracts of root and bark gave positive result for some phytochemicals (Saponin, flavonoids, reducing sugar, carbohydrates, volatile oils, phenolic compounds) (Tab. 6).

Table 6 Summary of the result from the phytochemical analysis of aqueous and hexane extracts of root and bark of *Dennettia tripetala*

Phytochemical constituents	Aqueous		Hexane	
	Bark	Root	Bark	Root
Alkaloids	-	-	-	-
Saponins	+	+	-	-
Tannins	-	-	-	-
Volatile oils	+	+	-	-
Anthracene	-	-	-	-
Flavonoids	+	+	-	-
Reducing sugars	+	+	-	-
Carbohydrates	+	+	-	-
Cyanogenetic glycerides	-	-	-	-
Phenolic compounds	+	+	-	-

\+ - Present.
\- Absent.

The bacterial isolates all displayed an MIC value against hexane and aqueous root and bark extracts. The minimum inhibitory concentration of the aqueous extract of root to inhibit the bacteria test organisms ranged from 50 - 400 mg/ml. The lowest MIC of 50 mg/ml was recorded against *S. aureus, B. subtilis, E. faecalis* and *P. aeruginosa* while the highest MIC of 400 mg/ml was recorded against *E. coli, S. aureus* and *E. feacalis.*

Table 7 The minimum inhibitory concentration of aqueous extract of the root of *D. tripetala* on test organisms.

Test organisms	Concentration of extracts (mg/ml)			
	Aqeous		Hexane	
	Root	Bark	Root	Bark
Staphylococcus aureus	50	100	400	400
Bacillus subtilis	50	100	100	200
Escherichia coli	100	100	200	400
Enterococcus faecalis	50	50	200	400
Pseudomonas aeruginosa	50	50	200	200
Candida albicans	>400	>400	>400	>400
Microsporum audouinii	>400	>400	>400	>400

DISCUSSION

The aqueous preparation of *D. tripetala* roots exhibited a greater antibacterial activity in comparision with other extracts prepared from the same plant. The antimicrobial activity of *D. tripetala* root can be attributed to the metabolites which it contains. It was observed that sensitivity increased with increasing concentration of the aqueous root extract. The hexane extracts showed little or no activity against the test microorganisms. This might be attributable to the fact that there was no phytochemicals extracted for it to show activity against the test microorganisms. The potency of the extract was comparable to those of antibiotics which are pure substances. The extracts did not show any activity against fungi (i.e. no antifungal activity) probaably due to the high resistant nature of the fungal strain used. The antifungal agent, fluconazole did not show activity against the fungal isolates probably because the strains of the fungi used are resistant to the activity of the antibiotic.

Based on the statistical analysis, there was significant difference between the inhibitory effect of the aqeous root and bark extracts against *P. aeruginosa*, *B. subtilis, E. coli, S. aureus, E. faecalis*. However, there was no significant difference between the inhibitory effect of the hexane bark and root extract. Considering the minimum inhibitory concentration (MIC) values of the aqueous root extract of the plant, the most potent activities were against *Staphylococcus aureus, Bacillus subtilis, Escherichia coli, Enterococcus faecalis* and *Pseudomonas aeruginosa*. The ability of the extract to inhibit some of the test bacterial isolates further confirm its traditional medical use by the traditional medical practitioners for the treatment of stomach disturbance and skin infections. *Staphyloccocus aureus* is known to play a significant role in skin diseases including superficial and deep follicular lesion, so the strong activity of the aqueous root extract of *D. tripetala* indicated that it could be effective against skin diseases. The antimicrobial activity observed for the aqueous extract of the root may be due to the fact that roots store more of the chemical compounds produced by plants than the bark. The root has cells that function in storing or accumulating certain active secondary metabolites and also due to the solvent used in extraction (i.e. distilled water) which is a polar solvent. However, the mechanisms through which the extract from the present study exert its antimicrobial activities requires further elucidation. The hexane extracts of the root and bark showed no activity against the test organisms.

A great number of chemicals found in plants have been said to be responsible for the medicinal properties of the plants. Saponins have been known to provide the starting material for the synthesis of corticosteroids and oral contraceptives **(Trease and Evans, 1996),** which are drugs that affect the female hormones. This observation supported the usage of *D. tripetala* in preparation of dishes for pregnant and postpartum women and also in prevention of nausea in pregnant women according to **Nwinuka and Nwiloh (2009)**. Volatile oil (i.e. B-phenylnitroethane) present in the plant parts, gives it its typical fragrance as well as for its pungency. These volatile oils together with phenolic compounds have antibacterial property which supports the use of the plant as antibacterial. Flavonoids are strong antioxidants, also found to be effective antimicrobial substances *in vitro* againsta wide range of microorganisms by inhibiting the membrane bound enzymes **(Cowan, 1999).** This support the use of the plant as antibacterial drugs. **Ejechi and Akpomedaye, (2005)** reported that essential oil and phenolic acid of pepper fruit can play a significant role in food preservation and protection against pathogens when they tested the extracts on fresh beef. The findings in this study agree with earlier studies that, not all phytochemicals are present in all plant parts and that those present differ according to the type of extracting solvent used **(Ayinde *et al*., 2007 ; Tijjani *et al.,* 2009).** Based on this, **Okwu *et al.,* (2005**) using ethanolic extract of the fruit of *D. tripetala* isolated phenanthrene alkaloid; uvariopsine, which was the first report of isolation of uvariopsine from the family Annonaceae. However, in the present study, the aqueous and hexane extracts did not show the presence of alkaloids (Table 6).

CONCLUSION

The presence of the phytochemicals in the aqueous extract of root has shown that *D. tripetala* root can be very effective against some bacteria making it a good antibacterial agent. The aqueous extract of the root contained active phytochemicals as measured by the degree of inhibition and number of microorganisms inhibited. Therefore it may be considered as potential antimicrobial agents for use in food products and post harvest disease control. However, further studies are recommended on the chemical characterization as well as bio-activity guided studies of the aqeous extract of the root.

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FUNGAL CONTAMINATION AND MYCOTOXIN PRODUCTION BY *ASPERGILLUS* SPP. IN NUTS AND SESAME SEEDS

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ABSTRACT

This work reports the occurrence of the fungal flora and evaluates the mycotoxigenic potential of *Aspergillus* genera in 63 samples of oil seeds and nuts (almonds, pistachio and sesame seeds). Fungal isolation and identification revealed the presence of 5 genera (*Aspergillus*, *Penicillium*, *Cladosporium*, *Mucor* and yeasts) with the predominance of *Aspergillus* section *Nigri*. A number of 138 strains of *Aspergillus* section *Nigri* and 91 of *Aspergillus* section *Flavi* were isolated and tested for their ability to produce ochratoxin A (OTA) and Aflatoxin, respectively. The detection of Aflatoxins and OTA production was carried out using thin-layer chromatography (TLC). Our results showed that 93.93% of *Aspergillus carbonarius* were able to produce OTA, but none of the *Aspergillus niger* aggregate was found to be an OTA-producer. Among the 91 *Aspergillus* section *Flavi* isolates, 88 were identified as *Aspergillus flavus* and 3 as *Aspergillus parasiticus*. All *A. parasiticus* were strong AFB and AFG producers. A percentage of 30.3% of *A. flavus* isolates produced AFB1, with levels ranging from 0.69 to 44.28 $\mu g.g^{-1}$. The frequency of aflatoxigenic *A. flavus* strains was higher in pistachios (46.3%) than in almonds (30.3%) or sesame seeds (23.52%). Cyclopiazonic acid (CPA) and sclerotia production were carried out on CYA medium. All aflatoxigenic *A. flavus* strains produced CPA, whereas 29.2% produced sclerotia (L-type).

Keywords: *Aspergillus*, aflatoxin, ochratoxin A, cyclopiazonic acid, sclerotia

INTRODUCTION

Mycotoxins are toxic secondary metabolites produced primarily by *Aspergillus*, *Penicillium* and *Fusarium* spp. under appropriate environmental conditions. They are considered to be a major factor in the spoilage of foodstuffs, leading to great economic loss and a major public health hazard **(Dwivedi *et al.*, 1984)**. Human exposure to mycotoxins is difficult to avoid because *Aspergillus* grows aggressively in many commodities and at all stages of the food chain: in the field and during storage or processing **(DeVries *et al.*, 2002).** Aflatoxins and ochratoxin A are toxins of serious concern witch are synthesized by several *Aspergillus* species and are highly toxic to humans and animals. AFs are synthesized by species of *Aspergillus* section *Flavi* and especially by *A. flavus* and *A. parasiticus*. The most common and toxic aflatoxins (AFs) naturally occurring are AFB1, B2, G1 and G2 **(Astoreca *et al*, 2011).** They have been clearly identified as highly toxic, mutagenic, teratogenic, and carcinogenic compounds and have been implicated as causative agents in human hepatic and extrahepatic carcinogenesis **(Massey *et al.*, 1995).** The International Agency for Research on Cancer has classified naturally occurring mixtures of aflatoxins as carcinogenic to humans (Group 1) **(IARC 2002).** Many foods and feeds can become contaminated with aflatoxin; however, the most pronounced aflatoxin contamination is in cereals, nuts, cotton, fig, spice and coffee **(Freitas and Brigido, 1998).** Ochratoxin A is a nephrotoxic mycotoxin produced by several species of the genera *Aspergillus* and *Penicillium*. The occurrence of ochratoxin A on food commodities such as grapes or grape products, spices, coffee and cocoa is mainly due to the presence of *A. ochraceus*, *A. carbonarius* and *A. niger* **(Schmidt-Heydt *et al.*, 2011).** The co-occurrence of different mycotoxins increases the probability of synergistic effects, which can increase the risk to human health. In addition to AF, some *A. flavus* strains produce other mycotoxins such as cyclopiazonic acid (CPA). CPA is an indole tetramic acid that is toxic to animals and humans. CPA-producing fungi can grow on many substrates, including cheese, meat products, and various grains and seeds **(Chang *et al*, 2009).** Co-contamination of food commodities by CPA and carcinogenic aflatoxins has been reported in different geographic areas **(Horn and Dorner, 1999; Martins and Martins, 1999; Fernandez-Pinto *et al.*, 2001).** The toxicity of CPA in many animal species has been studied. It causes weight loss, diarrhea, degeneration and necrosis of the muscles and viscera, convulsion and death **(Purchase, 1971; Dorner *et al.*, 1983; Nuehring *et al.*, 1985).** The aim of the present work is the study of fungal contamination and mycotoxins (aflatoxins, ochratoxin A and CPA) production by *Aspergillus* strains isolated from several samples of stored oilseeds in Algeria to provide useful information.

MATERIAL AND METHODS

Sampling

A total of 63 samples were investigated in this study. Samples were randomly collected between (April-July) 2011 from different markets and shops in north Algeria (sample sizes of 200 g). The selected commodity groups were: pistachio (15 samples), almonds (36 samples) and sesame seeds (12 samples). Samples were stored in plastic bags at 4°C until the analysis.

Reagents

All reagents (potassium chloride, phosphoric acid, hydrochloric acid, ammonium hydroxide, β-cyclodextrin) were of PA grade. Deionized water was used for the preparation of all aqueous solutions. Mycotoxin standards (aflatoxins, ochratoxin A and cyclopiazonic acid) and Ehrlich's reagent (4-dimethyl-aminobenzaldehyde) were purchased from Sigma Aldrich (France). All other solvents and reagents were of analytical grade purchased from Merck, Germany.

Fungal isolation and identification

Dilution plating was used as the enumeration technique **(Pitt and Hocking, 1997).** Ten grams of each grounded sample were dispersed in 90 mL of 0.05% Tween 80 sterile distilled water. Decimal dilutions (up to 10^{-3}) were prepared and 0.1 mL of each dilution was inoculated in duplicate on DRBC (Dichloran Rose-

Bengal Chloramphenicol Agar) medium. All plates were incubated for 5 days at 28°C. The concentrations of fungi were expressed as colony forming units per g (CFU/g). After incubation species belonging to the genera of *Aspergillus* were isolated and identified. For morphological identification, isolated species were sub-cultured on three media: Czapek Yeast Agar (CYA), *Aspergillus flavus* and *parasiticus* agar (AFAP) and Czapek agar (CZ). Identification was performed according to standard taxonomic systems based on color of colony, the shape of conidiophores and conidia's dimension examined microscopically **(Pitt and Hocking, 1997; Klich, 2002).** Identification of *Aspergillus* section *Flavi* was completed by taking into account a combination of all the observed criteria, including, sclerotial production and AFs and CPA profiles.

Aflatoxigenic ability of the isolates

Aspergillus section *Flavi* isolates were maintained on plates containing Coconut agar medium (CAM) **(Davis *et al.*, 1987).** On this medium, aflatoxin-positive isolates showed a blue or blue-green fluorescence in agar surrounding the colonies under UV light. Inoculations were done by conidial transfer to center of plates containing (CAM). The plates were incubated at 28 °C in the dark for 7 days. The reverse side of colonies was periodically observed under long-wave (365 nm) UV light. Cultures were examined daily (for 1 week) under the UV light for detection of florescence signal of aflatoxigenic isolates. A blank consisting of sterilized, non-inoculated CAM medium, incubated under the same conditions, was used as control. To confirm the correlation between fluorescence and aflatoxin production, all colonies, whether or not they showed fluorescence, were extracted according to the method described by **(Filtenborg *et al.*, 1983).** The agar plug methods for extracellular and intracellular mycotoxins were used for toxin extraction of the colonies. Three agar plugs of the solid medium were removed from different points of the colony for each culture, weighted and collected into small tubes. Extraction was done by adding a volume of 1mL of methanol to each tube **(Bragulat*et al.*, 2001).** After incubation for 1 hour at room temperature, all tubes were centrifuged at 13000 rpm for 10 min. The obtained extracts were filtered through a 0.45 µm Millipore filter then analyzed by TLC. Quantification of aflatoxin B was done by HPLC.

CPA production

The strains were tested for cyclopiazonic acid in Czapek Yeast Agar medium (CYA: Sucrose 30 g/L, Powdered Yeast Extract 5 g/L, $K2HPO_4$ 1 g/L, $NaNO_3$ 2 g/L, KCl 0.5 g/L, $MgSO_4 \cdot 7H_2O$ 0.5 g/L, $FeSO4 \cdot 7H_2O$ 0.01 g/L, $ZnSO_4 \cdot 7H_2O$ 0.01 g/L, $CuSO_4 \cdot 5H_2O$ 0.005 g/L, Agar 20 g/L). All strains were inoculated on 6 cm diameter plates and incubated at 28 °C for 14 days, in the dark **(Gqaleni *et al.*, 1997).** The experiment was carried out with two replicates. Following incubation, the methodology of **Bragulat *et al.* (2001)** described previously was employed. The obtained extracts were analyzed using thin-layer chromatography (TLC).

Detection of aflatoxins and CPA production by TLC

The analysis of aflatoxins and CPA production of all *Aspergillus* section *Flavi* was carried out on a silica gel 60 plate 20 × 20 cm (Merck). For aflatoxins, chloroform: acetone (90:10, v/v) was used as developing solvent. 20 µL of 1 $\mu g.mL^{-1}$ concentration of B1, B2, G1 and G2 aflatoxins and 20 µL of test samples were spotted on TLC plates and run for 45 min in a TLC tank. Plates were air dried and observed under UV light (365 nm) for presence or absence of fluorescent spots as well as their intensity. The detection of CPA was performed using Ethyl acetate/propanol/ammonium hydroxide (40:30:20) as developing solvent system **(Fernandez Pinto *et al.*, 2001).** The plates were dipped first in a 2% solution of oxalic acid in methanol for 2 min. 20 µL of test samples and CPA standard were spotted on TLC plates. After plates development CPA was visualized in daylight by treatment of the plates with Ehrlich's Reagent (1 g of 4 dimethyl amino benzaldehyde in 75 mL ethanol and 25 mL concentrated HCl) and appeared as a blue-purple spot.

HPLC analysis of AFB

The quantitative determination of AFB produced by aflatoxigenic isolates of *Aspergillus flavus* was performed with HPLC. The HPLC apparatus was a Shimadzu Liquid Chromatograph AD-0012-LC equipped with a Post-column Bromination (Coring Cell) and a fluorescence detector (365 nm excitation wavelength; 435 nm emission wavelength), Chromatographic separations were performed on a reverse phase C18 column (250 x 4 mm, 3 µm particle size). The system was run using a mobile phase containing distilled water, acetonitrile, methanol (6:2:2, v/v/v) with 119 mg/L of KBr and 110 µL/L of 65% HNO_3 under isocratic elution with a flow rate of 1 mL/min. The injection volume was 20 µL.

Sclerotial characterization

To assay for sclerotia production, plates containing CYA medium were inoculated with *Aspergillus* section *Flavi* mycelia obtained from a culture of 7 days in Potato dextrose agar medium (PDA). Cultures were incubated in darkness for 21 days at 30°C. Sclerotia were obtained by pouring 10 mL of water with Tween 80 (0.01%) per plate and scraping the surface of culture plates (two replicate plates per isolate) over Whatman filter paper and rinsing with tap water to finally air-dried **(Novas and Cabral, 2002)**. Formation of sclerotia was confirmed visually and selerotia type of each culture was confirmed by measuring their size. The sclerotia isolates were classified according to the sclerotia size; L strain isolates produced very few sclerotia with diameter greater than 400 µm, and S strain isolates produced numerous sclerotia with diameter under 400 µm **(Cotty, 1989).**

Ochratoxin A production and analysis

The production of OTA was studied on 105 strains of *Aspergillus niger* aggregate and 33 of *A. carbonarius*. All isolates were cultured on CYA medium for 7 days at 28°C. OTA was extracted by the agar plug method (previously described). OTA production was detected in the extracts by thin layer chromatography (TLC) using toluene/ethyl acetate/ 90% formic acid (5:4:1, v/v/v) as developing solvent.

RESULTS

Fungal contamination

Significant differences were observed between the frequency of fungal isolates in almonds, pistachio and sesame seeds. Almonds were the most contaminated raw material (8.05×10^3 CFU/g) followed by pistachios (6.85×10^3 CFU/g) and sesame seeds (2.95×10^3 CFU/g). Mycological analysis showed that all samples were contaminated by fungi, excepted salted almonds (8 samples). Five genera were isolated from different nuts and sesame seeds. The frequencies of isolated fungi from different nuts were *Aspergillus* (84.69%), *Penicillium* (9.31%), *Mucor* (4.48%), *Cladosporium* (0.84%) and yeasts (0.68%). *Aspergillus* was the most common genus of fungi and was represented by species including: *A. flavus*, *A. parasiticus*, *A. niger* aggregate, *A. carbonarius* and *A. ochraceus.* Predominant mycobiota belonged to the genera *Aspergillus* section *Nigri* and were represented by *A. carbonarius* and *A. niger* aggregate (Tab 1). These two species were differentiated by their conidia size. *A. niger* aggregate and *A. flavus* were found in 76.66% and 53.33% of samples, respectively, *Aspergillus niger* aggregate was the most dominant.

Table 1 Abundance and distribution of *Aspergillus* isolates in the different samples

Sample	Almond	Sesame seeds	Pistachio	Isolates tested
A. flavus	5.7%	6.37%	25.03%	88
A. parasiticus	0.81%	0.63 %	1.21%	3
A. niger aggregate	76.71%	46.08%	72.89%	105
A. carbonarius	2.54%	17.85%	-	33
A. ochraceus	-	0.8%	-	-

***Aspergillus* section *Flavi* identification**

Aspergillus section *Flavi* isolates were identified using morphological characters (mainly colony color on CZ, CYA and conidia morphology) by comparison to reference strains of *A. flavus*, *A. parasiticus* and *A. nomius.* Ninety one isolates belonging to *Aspergillus* section *Flavi* were obtained exclusively from almonds (33), pistachio (41) and sesame seeds (17). *Aspergillus* strains were classified in two groups: isolates with dark-green colonies and rough conidia, which were classified as *A. parasiticus* (3 isolates), and isolates with yellow-green colonies and smooth to finely rough globose conidia, classified as *A. flavus* (88 isolates). *A. flavus* (producing aflatoxin B) and *A. parasiticus* (producing aflatoxins B and G) were found together on all samples and they showed a bright orange color of the colony reverse on AFPA.

Analysis of aflatoxins

Among the 91 isolates of *Aspergillus* section *Flavi* tested for aflatoxins production on CAM, 26 isolates (28.59%) showed fluorescence in this medium after 5-7 days of incubation. These results were in concordance with those obtained from the methanol extracts from all strains cultivated in CA medium. Aflatoxins production led to the development of fluorescent area around the colonies under UV light. None of the fluorescent isolates were found to be unable to produce aflatoxins under the same conditions. TLC analysis of the extracts showed that, 33 isolates (36.26%) were aflatoxin producers**.** Thirty isolates were identified previously as *A. flavus* produced only aflatoxin B, while *A. parasiticus* isolates (3 isolates) were aflatoxinogenic and were capable to produce both aflatoxins B1 and G1. All strains producing a strong blue fluorescence on CAM after 5 days of incubation showed a high AFBs production whereas those with a weak fluorescence on CAM, detectable after 7 days of incubation were weak

producers**.** The quantitative analysis of aflatoxin by HPLC showed concentrations of AFB1 ranging between 0.69 $\mu g.g^{-1}$ to 44.28 $\mu g.g^{-1}$and lower levels of AFB2 ranging from 0.2 $\mu g.g^{-1}$ to 6.05 $\mu g.g^{-1}$ (Tab 2).

Table 2 Aflatoxin-producing ability of isolated *Aspergillus* section *Flavi* strains.

Species	Total strains (%)	AFB1 (µg/g) Positive strains (%)	AFB2 (µg/g) Positive strains (%)
A. flavus	88 (96.7)	0.69– 44.28 (34.09)	0.2- 6.05 (30.68)
A. parasiticus	3 (3.3)	53.2– 54.35 (100)	2.14- 3.98 (100)

Sclerotial and CPA production

The sclerotia had a nearly spherical shape with irregular margins, strongly dense and hard, at first creamy white in color and blackish gray over time. Fifteen strains (16.48 % of total strains) were able to produce only large sclerotia (L). 24.24% (8 isolates) of aflatoxigenic *A. flavus* isolates were found to be sclerotia producers. All aflatoxigenic isolates produced also CPA. However, *A. parasiticus* strains were strong AFB and AFG producers, but did not produce detectable CPA and sclerotia.

Identification of chemotypes

The strains were classified into chemotypes based on AFs and CPA production patterns (Tab 3). Atoxigenic isolates were the most represented chemotype (65.9%). No strains were found able to produce AFB, AFG and CPA. 34.09% of *A. flavus* produced AFB and CPA and were included in a different chemotypes. *A. parasiticus* isolates produced both AFB and AFG but were not able to produce CPA.

Table 3 Incidence of chemotypes of *Aspergillus* section *Flavi* based on mycotoxigenic profile (AFs and CPA) **(Vaamonde *et al*., 2003).**

Chemotype	Mycotoxins			Number of isolates of each chemotype (%)
	AFB	AFG	CPA	
I	+	-	+	30(32.96%)
II	+	+	+	0
III	+	+	-	3(3.29%)
IV	-	-	+	0
V	-	-	-	58 (63.73%)

Legend: (+) – presence, (-) – absence.

Ochratoxin A production

The ochratoxiginity of *A. carbonarius* and *Aspergillus niger* aggregate strains from the methanolic extracts was determined by TLC. Our results showed a high production percentage within *A. carbonarius* isolates, indeed, 93.93% (31 isolates out of 33) were able to produce OTA. In the other side all strains that belong to *A. niger* aggregate (105 isolates) were found to be non-producers of OTA.

DISCUSSION

Food products may become contaminated by fungi that can be responsible of damaging them. Contamination of edible greasy seeds by fungi, mostly pistachio and almond, were reported in different countries. Fungal identification is very important to provide information about which mycotoxins could be present **(Maenetje and Dutton, 2007).** To date, a small research has been done in this area in Algeria. This work had been performed to investigate the contamination of some oilseeds. Total fungal counts showed a good quality of the studied food products with acceptable contamination levels ranged from $2.95x10^3$ to $8.05x10^3$ CFU/g **(Andrews, 1992).** The assessment of the incidence of fungal flora revealed that (80%) of grain samples contained more than one species of fungi. Aspergilli were the major species most commonly isolated in all products. The incidence of *Aspergillus* section *Nigri* was higher than those belonging to *Aspergillus* section *Flavi*. This species is a very frequent fungal contaminant found worldwide on various substrates such as cereals, grapes, coffee bean and nuts. Fungal and mycotoxins contaminations vary depending on the climate. In Algeria, climatic conditions characterized by high humidity and temperature and inadequate storage practices contribute to increase the potential for contamination of the commodities by *Aspergillus* and their toxins **(Riba *et al.*, 2010).** *Aspergillus* and *Penicillium* species mainly grow during storage. The predominance of *Aspergillus* in stored nuts bean has been reported by several authors **(Adebajo and Diyaolu, 2003; Rostami *et al.*, 2009).** Salted almonds (8 samples) instead, did not show any fungal contamination. This can be explained by the strong activity of salt against fungal growth. Similar results were obtained by other workers including **Thamaboripat *et al.* (1992)**; they reported that high concentrations of NaCl may affect the water activity required for fungal growth.

From all the analyzed samples 91 strains of *Aspergillus* section *Flavi* were tested for their ability to produce aflatoxins on Coconut Agar Medium (CAM). The presence of fluorescence on this medium was correlated with AFs production at a very high level on TLC and HPLC.

On the basis of sclerotial size *A. flavus* isolates can be divided into two subtypes (L-type and S-type). Many studies attempt to found a relationship between aflatoxins production and sclerotial phenotype. Some showed a positive interrelationship between regulation of aflatoxin biosynthesis and the production of small sclerotia **(Novas and Cabral, 2002; Pildain *et al.*, 2004).** Whereas, others observed that the L strains produce higher levels of aflatoxins than the S strain isolates **(Astoreca *et al.*, 2011; Abbas *et al.*, 2005).** In our survey, sclerotia were all of the L-type (> 400 µm), and were present in 29.2% of the *A. flavus* isolates. Small sclerotia production was not found. No correlation could be established between sclerotia presence/size and toxigenicity in this case. Abundance of L strains was reported in many previous studies **(Astoreca *et al.*, 2011; Donner *et al.*, 2009; Giorni *et al.*, 2007).** The conditions responsible for the distribution of *A. flavus* S- strains still unknown and appear to be complex. Environmental factors are known to affect sclerotial biogenesis. For example, in the Eastern Province of Kenya the domination of S-strains was observed **(Probst *et al.*, 2007; Pildain *et al.*, 2008).** Recently, **Riba *et al*. (2010)** reported a dominance of L-strains in Algerian wheat simples. These results contrast with those reported in our investigation, showing a positive correlation between the distribution of these two different types and the environment factors.

The mycotoxigenic potential and profile of *A. flavus* is known to be variable. This species has been frequently divided into groups, depending on their toxigenic profile **(Razzaghi-Abyaneh *et al.*, 2006; Vaamonde *et al.*, 2003).** In our work, more than half isolates (65.9%) are atoxigenic. Analogous results were reported in many studies by several authors showing a high percentage of these isolates in various foodstuffs **(Horn and Dorner, 1999; Razzaghi-Abyaneh *et al.*, 2006; Sanchez-Hervas *et al.*, 2008).** In general, the incidence of atoxigenic strains of *Aspergillus* section *Flavi* communities varies with geographic origin **(Atehnkeng *et al.*, 2008; Pildain *et al.*, 2004)** and substrate **(Vaamonde *et al.*, 2003).** In the present study, *A. flavus* isolated from Pistachio showed a high aflatoxins production (46.3%), than those isolated from almonds (30.3%) and sesame seeds (23.52%). Atypical *A. flavus* isolates (Group II), which produce small sclerotia and AFB, AFG and CPA have not been identified in our survey. This kind of isolates is intermediate between *A. flavus* and *A. parasiticus*. This phenotype has been previously isolated in soil and agricultural samples from West Africa, Argentina and Australia and has been classified as *A. minisclerotigenes* **(Pildain*et al.*, 2008).** 30 strains (34.09%) of *A. flavus* were capable to produce both CPA and AFB (chemotype III). This type of isolates belongs to typical *A. flavus* isolates. The co-occurrence of aflatoxins with cyclopiazonic acid has been reported in peanuts, corn and animal feeds in different geographic areas **(Uranoet *al.*, 1992; Horn and Dorner, 1999; Martins and Martins, 1999; Fernandez-Pinto *et al.*, 2001).** *A. parasiticus* strains are more uniformin their toxigenic abilities: they are usually reported as strongly aflatoxigenic **(Horn *et al.*, 1996; Razzaghi-Abyaneh*et al.*, 2006; Tran-Dinh*et al.*, 1999).** They are also known to be producers of B- and G-type aflatoxins but never CPA. All *A. parasiticus* isolates were found to be strongly aflatoxigenic and produce both AFB and G.

Black aspergilli were tested on CYA medium for ochratoxin A production. None of *A. niger* aggregate produced ochratoxin A, but 31 of 33 isolates of *A. carbonarius* were able to produce this toxin. Among the species belonging to the *Aspergillus* genera (*A. niger*, *A. ochraceus* and *A. carbonarius*) *A. carbonarius* is the main OTA-producers. Strains of *A. niger* are considered as weak OTA-producers **(El Khoury and Atoui, 2010). Battilani *et al.* (2003)** found that the most ochratoxigenic strains isolated from grapes belonged to *A. carbonarius*. Also, this species is the most probable source of OTA in dried vine fruits **(Abarca*et al.* 2003).** Our findings are in agreement with most other studies on these species. **Battilani *et al.* (2006)** and **Perrone *et al.* (2006)** reported high percentage of OTA producers on *A. carbonarius* (from 70-100%), although, *A. niger* aggregate, the most common, showed a low percentage (20%) of OTA producing strains.

CONCLUSION

This research has provided information about contamination by molds and their mycotoxins in some Algerian greasy edible seeds. A very high development of *Aspergillus* genus has been noted. A study of the aflatoxigenicity of 91 *Aspergillus* section *Flavi* isolated and identified by a combination of morphological criteria, and mycotoxin profiles, revealed that all *A. parasiticus* strains were aflatoxigenic and produce both AFB and AFG, whereas 30.3% of *A. flavus* isolates produced only AFB. The aflatoxigenicity of these isolates was variable in all samples. In almonds and sesame seeds a development of *A. carbonarius* has been observed. This species was found to be highly ochratoxigenic. The presence of species like *A. flavus*, *A. parasiticus* and *A. carbonarius*, can lead to aflatoxins and OTA production in foodstuffs. To minimize fungi contamination and manage the risk of mycotoxins production, good storage practices and control measures should be established because it still the most effective tool to use.

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DETERGENT COMPATIBLE COLD-ACTIVE ALKALINE AMYLASES FROM *CLAVISPORA LUSITANIAE* CB13

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ABSTRACT

After two stages of screening, five environment isolates of yeasts showing amylolytic activity at alkaline pH and residual activity at cold temperatures were isolated from rotten vegetables. Based on pH and temperature robustness, amylases from CB13 were selected for further characterization. The enzymes from this yeast-isolate showed optimum activity at pH 11 and temperature 40ºC, 42 % residual activity at 4ºC, no dependence on Ca^{2+} for activity and stability, resistance to EDTA and SDS, and amplification in activity in presence of Mn^{2+} and Co^{2+}. The amylolytic activity was thermostable showing retention of 50% of optimum activity after boiling for 30 min. The enzymes retained nearly 80% of activity after exposing to various detergent components, and also to commercially available laundry detergents for 2h. The morphological, physiological and molecular characterization of the isolate CB13 led to its identification as *Clavispora lusitaniae*. This is the first report on the screening of detergent compatible α-amylase with residual cold-activity from yeast isolated from rotten vegetables.

Keywords: *Clavispora lusitaniae*, Alkaline α-amylases, Detergent compatible α-amylases, Cold-active α-amylase

INTRODUCTION

Microorganisms from nature have been found to contain a great variety of enzymes with tremendous variation in kinetic features, suitable for applications in diverse types of industries (**Sidhu *et al*., 1997**). Among various enzymes of industrial importance, α-amylase (EC 3.2.1.1) holds top position accounting for approximately 25% of the enzyme market (**Sidhu *et al*., 1997**). Most importantly, they find applications in starch processing, brewing and sugar manufacturing, desizing in textile industries and detergent manufacturing (**Pandey *et al*., 2000**). The α-amylases have been commercially produced applying bacteria belonging to the genus *Bacillus* such as *B. amyloliquefaciens*, *B. stearothermophilus*, *B. subtilis* and *Bacillus licheniformis* (**Sivaramakrishnana *et al*., 2006**).

Until now, there has been a wide interest in thermostable enzymes including thermostable α-amylases for obvious reasons (**Arikan, 2007; Carvalho *et al*., 2008**). It is recently, the cold-active enzymes have started arousing much industrial interest because of potential economic benefits such as less energy investment, less chance of contamination (**Cavicchioli *et al*., 2002**) and higher quality of products (**Sahay *et al*., 2012; Sahay *et al*., 2013**) as a result of cold processing that goes with these enzymes. Thus for certain industries such as those of food, detergent etc, the cold active enzymes are now considered to be highly desirable (**Nakagawa *et al*., 2002; Roohi *et al*., 2013; Sahay *et al*., 2013**). One of the essential characteristics of cold-active enzymes viz., thermal instability is however a major hindrance in certain application such as detergent. Amylases are one of the highly used enzymes in the formulation of enzymatic detergent, and 90% of all liquid detergents contain these enzymes (**Hmidet *et al*., 2009**).

The enzymes, amylases have many areas of applications; each application requires a special set of properties with respect to thermostability, pH profile, pH stability, Ca^{+2}-independency etc. For example, amylases used in starch industry must be active and stable at low pH but those used in detergent industry must be active and stable at high pH, Ca^{2+}-independent and resistant to detergent components such as certain surfactants and oxidizing, chelating and bleaching agents (**Carvalho *et al*., 2008; Chakraborty *et al*., 2012**). It is, therefore, important to explore various sources to isolate such microbe as producing amylases with desirable features for specific application. The psychrotrophic microbes so far yielded very unstable amylases with hardly desirable features for application to detergent formulation (**Roohi *et al*., 2013**). The present paper describes isolation and partial characterization of cold active alkaline α-amylases from a mesophilic natural yeast for application in detergent industry.

MATERIALS AND METHODS

Site, sampling and source materials

Various rotten vegetables were collected from the local market of Bhopal (34.5°–36° South latitude and 70°–66.5°Western longitude) and carried to laboratory aseptically. For inoculums preparation, vegetables after cutting into pieces of about 1mm^2 with sterile knife were placed in flasks containing 10 ml of sterile saline water (0.1%) to a final concentration of 0.1 gm ml^{-1}. The sample was shaken at165 rpm for 1 h at room temperature. Aliquot of 0.1 ml size was spread on the surface of PDA (potato, glucose, agar) medium supplemented with chloramphenicol in the concentration of 1mg ml^{-1}. The plates were incubated at 25°C for 72 h.

Screening of extracellular amylase secreting isolates

Medium used was a modified minimal synthetic medium (MM) containing (g l^{-1}); $(NH_4)_2SO_4$-5, KH_2PO_4-1, NaCl-0.1, $MgSO_4$-0.5, $CaCl_2$-0.01, starch-0.1, Agar-15, and pH adjusted to 7.0 (**Sahay *et al*., 2013**). The MM contained starch as sole carbon source to screen yeast isolates exhibiting constitutive amylolytic activity. The plates were incubated at 25ºC for 48h. Amylase production was detected after flooding the plates with lugol's iodine (**Arikan, 2008**).

Second screening

In the second round of selection, the main parameters used were the rate of colony-growth and ability to grow at higher pH. The medium used was MM with pH adjusted to 7.0 and 9.0 with 0.1 N NaOH. Since agar is not gelled at higher pHs properly, psyllium-gelled medium was also used (**Sahay, 1999**).

Enzyme production

One ml of log phase culture (density 10^6 l^{-1}) from yeast growth medium (0.67% yeast nitrogen base without amino acids and 2% glucose) was transferred to 250-ml conical flask containing 50 ml of the yeast production medium (0.67% YNB and 2% starch). The growth was carried out at 25ºC in a refrigerated shaking incubator (REMI/CIS 4) at 150 rpm, and was monitored by measuring OD_{600} of growth medium. Aliquots of 5 ml sizes were withdrawn at 12h intervals, cells were spun down at 5000 rpm for 10 min and the supernatant was used to assay α-amylases.

Partial purification

After 72h of growth, the culture was centrifuged at 5000 rpm to remove cells. The supernatant was saturated with ammonium sulphate to 40%, 50, 60%, and 80% level and precipitated proteins were collected by centrifugation at 10000 rpm at 4°C (REMI/CM12) for 10 min. Maximum activity was observed in 50% fraction, that was thus dialysed against 100 mM phosphate buffer (pH 7.0) at 4°C, resuspended in the same buffer in minimum volume. The protein was further purified by DEAE chromatography as earlier (**Roohi *et al*., 2013**) and stored at -10°C until used.

Enzyme assay

The reaction-mix containing 1 ml of 1% starch solution and 1 ml of properly diluted enzyme (kept at -10°C) was incubated at 25°C for 30 min. A 0.3ml aliquot of this solution was taken in another test tube, and equal volume of of 3,5-dinitrosalicylic acid reagent was added to it. The solution was then boiled for 5min, and then cooled down to room temperature. It was diluted with 2.7ml of distilled water and, absorbance was measured at 540 nm using UV-Vis spectrophotometer. One unit of amylase activity was defined as the amount of enzyme that released 1µM of reducing sugar equivalent to glucose per min under the assay condition (**Miller, 1954**). The experiments were performed in three sets of duplicate cultures, and the mean value of enzyme activity was determined.

Protein content

Protein concentration was determined by the method described earlier (**Lowry *et. al*., 1951**) using bovine serum albumin as standard.

Effect of pH

The effect of pH on the enzyme activity was determined by varying the pH of the reaction mixtures using the following buffers (100 mM): sodium acetate (pH 3.0-6.0), sodium phosphate (pH 6.0-7.0), Tris-HCl (pH 8.0-9.0) and glycine-NaOH (pH 10-11) followed by assay of the enzyme. The pH stability was determined by pre-incubating the enzymes in different buffers for 1h. The residual activity was then assayed in 100 mM sodium phosphate buffer (pH 7.0).

Effect of temperature

The effect of temperature on the enzyme activity was evaluated by measuring the amylolytic activity at different temperatures (4°C, 25°C, 40°C, 50°C) in 100 mM glycine-NaOH (pH 11). The effect of temperature on stability of α-amylases was determined by pre-incubating it in 100 mM glycine-NaOH (pH 11.0) at 50°C for 1h, 2h, 3h, 4h, 24h, 48h and 72h followed by measuring its residual activity. The enzymes were also tested for their stability at boiling temperature by boiling for 1min, 5 min, 10 min, 15 min, 20 min and 30 min followed by determining the residual activity.

Effect of metal ions and chelator

The effect of metal ions on amylases was determined by pre-incubation of the enzyme with various metal ions at a final concentration of 5 mM in glycine-NaOH buffer (pH 11.0), at 30°C for 30 min followed by their assay for the residual activity. Likewise, enzymes were pre-incubated with EDTA or SDS to the final concentration of 5 mM and 1% (w/v) respectively at 30°C for 30 min, to see their effect on amylolytic activity. To examine stability, the incubation period of the enzymes with above factors was extended to 60 min followed by their assay for the residual activity. The activity of the enzymes alone in 100 mM glycing-NaOH buffer (pH 11.0) was taken to be 100%.

Effect of detergent components

The effect of important detergent ingredients such as surfactants (SDS, Tween-80 and Triton X-100), and oxidizing (H_2O_2) and bleaching ($NaClO_3$) agents was studied by incubating enzyme (50 µl) with 950 µl of 1 % of these reagents in 10 mM glycine–NaOH buffer (pH 11.0) for 1h and 2h at 40°C (**Chakraborty *et al*., 2012**) before assaying the enzyme as compared to the control without any additive.

Effect of laundry detergents

The detergent brands used were Surf Excel® (Hindustan Unilever Limited-Mumbai, India) and Tide® and Ariel® (Procter and Gamble Home Products Ltd). They were diluted in double distilled water to a final concentration of 7 mg ml^{-1} to simulate washing conditions and heated at 100°C for 15 minutes to inactivate the enzymes that could be part of their formulation (**Carvalho *et al*., 2008**). The detergents were added to the reaction mixture and the reaction was carried out under standard assay conditions. To determine the stability of CB13 amylase in the presence of the different detergents, an amylase concentration of 1 $mg.ml^{-1}$ was added in detergent solution and incubated at 50°C for 12 h. Aliquots (0.5 mL) were taken at different time intervals and the residual activity determined at 20°C and 4°C and, compared with the control sample incubated at 50°C without any detergent (**Carvalho *et al*., 2008**).

Taxonomic characterization of CB13

Morphological and physiological characterizations of the most potential isolate, CB13 were carried out according to Barnet et al. (2000). All carbon sources except Arbutin, D-glucono-1,5-lactone, 2-keto-D-gluconate, 5-keto-D-gluconate, D-gluconate, D-glucuronate, D-glucarate and D-galactonate and nitrogen sources have been tested for their utilization by the yeast isolate CB13. For molecular characterization, DNA isolation from yeast was performed as per the protocol given earlier (**Harzu, 2004**). PCR amplification of D1/D2 region of the large ribosomal subunit was carried out applying primers NL-1 (5'-GCATATCAATAAGCGGAGGAAAAG-3') and NL-4 (5'-GGTCCGTGTTTCAAGACGG-3') and protocol given earlier (**Kurtzman and Robnett, 1997**). The purified ~600 bp PCR products were sequenced by automated DNA sequencer -3037xl DNA analyzer from Applied Biosystems using Big Dye® Terminator v3.1 cycle sequencing kit (Applied Biosystems). Sequence analysis software version 5.2 from applied biosystems was applied to align sequence data and generation of dendrogram. The sequences obtained for upper and lower strands were manually aligned before performing the analysis. BLASTN was used to compare this sequence to the non-redundant NCBI database to find the most similar sequence and were sorted by the E score. A representative sequence of 10 most similar neighbours was retrieved and multiple alignment was performed applying CLUSTAL W2 with the default settings. The evolutionary history was inferred using the UPGMA method (**Tamura *et al*., 2011**) and evolutionary analyses were conducted in MEGA5.

RESULTS AND DISCUSSION

Isolation of alkalitolerant amylase producing yeasts

In the first round, yeasts were isolated from various rotten vegetables showing constitutive amylolytic activity. In the second round, the selected yeasts were tested for their growth performance on alkaline medium and consecutively five yeasts showing constitutive amylolytic activity at pH 9 were selected (Tab 1) for further study. Amylases have been reported from a number of yeasts (**Wilson and Ingledew, 1982; Moranelli *et al*., 1982; DeMot and Verachtert, 1986; DeMot and Verachtert, 1987; Lefuji *et al*., 1996; Wanderley *et al*., 2004**), there is hardly any report of their isolation from *C. lusitaniae*. Amylases are one of the most important industrial enzymes finding application in a variety of fields. Bacteria are the most important organisms serving the source of industrial grade amylases so far (**Sajedi *et al*., 2005**), though enzymes with unique set of biochemical characteristics fit for specific applications have been reported from other classes of microbes (**Sivaramakrishnan *et al*., 2006**). As to the cold-active amylases, hardly any class of microbes other than bacteria of Antarctica origin has been explored (**D'Amico et al., 2003**). Therefore, cold-active amylases from these bacteria have become the model for biochemical study (**D'Amico *et al*., 2003**) and, contemplating future applications. Likewise, alkaliphiles have been isolated from various sources earlier (**Horikoshi, 1999**). We are reporting cold-active amylolytic activity with utterly novel features from a yeast-isolate, *C.lusitaniae* CB13 isolated from rotten cabbage.

Enzyme production

During growth on agar plates containing starch as substrate, selected yeast isolates formed large starch digesting halos around their colonies as were evident from staining with iodine solution. This was an indication of the presence of amylolytic activity. The degradation of starch was accompanied by cell growth and amylase secretion in the production medium. The extracellular amylolytic activity increased during cell growth and reached maximum values at 96 h of incubation, which continued till 120 h (data not shown). The optimum enzyme activity at 96 h as shown by CF2, SP1, SP3, CB13 and PTC were 41.6, 13.92, 41.6, 46.7 and 11.11 U/ml for 0.1 % substrate concentration respectively (Tab 1).

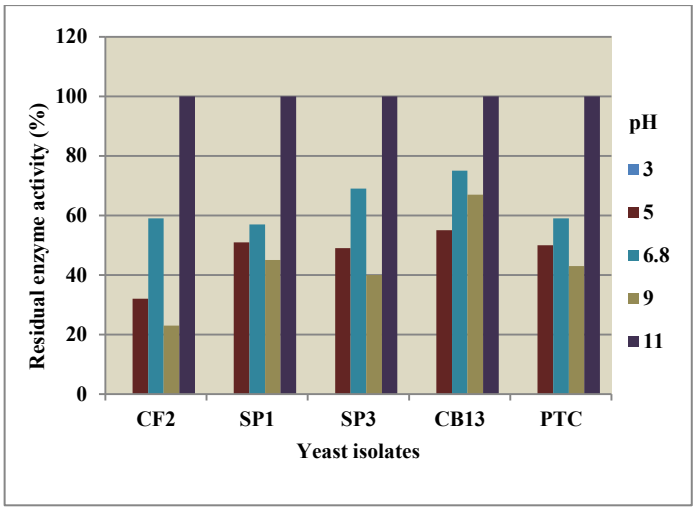
Table 1 Maximum activity, optimum pH and optimum temperature of amylases isolated from selected yeast isolates

Strains	Source	Maximum activity (U/ml/min)	Optimum pH	Optimum temperature (ºC)
CF2	*Brassica oleracea*	41.6	11	25
SP1	*Spinacia oleracea*	13.92	11	25
SP3	*Spinacia oleracea*	41.6	11	25
CB13	*Brassica oleracea* var. capitata	46.17	11	40
PTC	*Allium cepa*	11.11	11	40

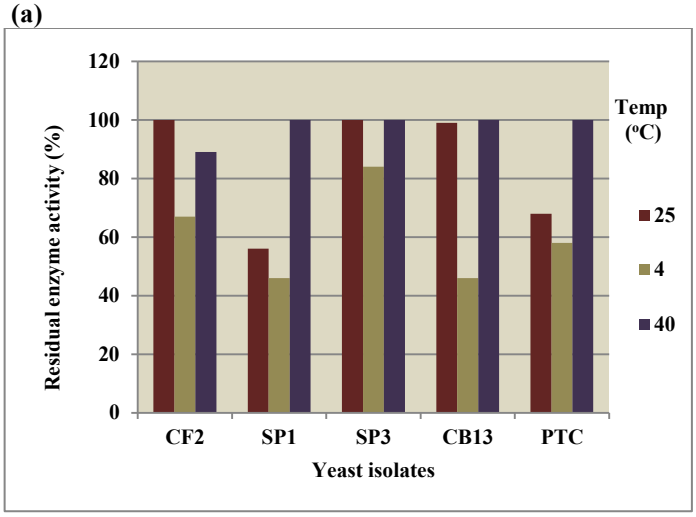
Preliminary characterization of enzyme from selected yeast-isolates

In case of most strains, 0.1% substrate concentration was found to be optimum. Only in case of CB13, a 0.2% substrate concentration was found to be optimum (data not shown).

The amylases from selected isolates were assayed at various pHs (Fig 1a). All the isolates seemed to yield alkaline amylases as the activity was found maximum at pH11. While CB13 seemed to be most pH-sturdy isolate since its activity profile fell to only 55 % while that of CF2 to 32% (Fig 1a). Overall, the amylases from all isolates showed one major peak activity at pH 11 and a minor one at near pH 6.8. The activity of enzyme (amylases) was also assayed at 4ºC, 25ºC and 40^{0}C and 50ºC. The amylases from all the isolates showed activity at all the temperatures though optimum temperature differ slightly, being 40^{0}C for all of them except CF2 (Fig 1b). The activity from all the isolates was found to continue down to 4^{0}C. The percentage of amylases activity at 4^{0}C varied from 84% (SP3) to 46 (SP1 and CB13) which is noteworthy.



(a)



(b)

Figure 1 Effect of pH (a) and temperature (b) on the extracellular amylolytic activity in the partially purified extract from five selected yeast-isolates.

Further characterization of amylase from CB13

The enzymes could resist boiling for 1 min, during which a loss of only 10% of its activity was recorded. The enzymes retained 20% residual activity after 30 min of boiling (Fig 2).

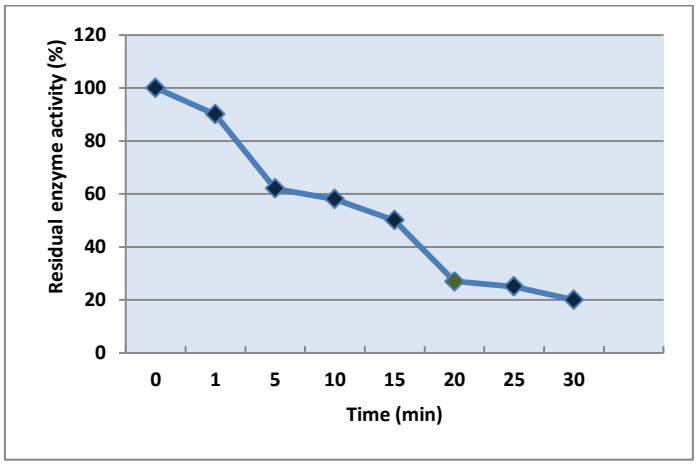


Figure 2 Effect of temperature (boiling temperature) on CB13 amylolytic activity

Ca^{2+} was found to have almost no effect on the enzymes immediately; though a little improvement of activity (8%) was observed after 1h of incubation with it. Among inhibitors, Cu^{2+} had drastic effect reducing activity to 16% whilst Fe^{3+} reduced the activity marginally by 10%. Among activators, Mn^{2+} was found to be the most effective one, followed by Co^{2+} and Mg^{2+} in that order. None of the metal ions tested was found to affect stability of the enzyme (Fig 3).

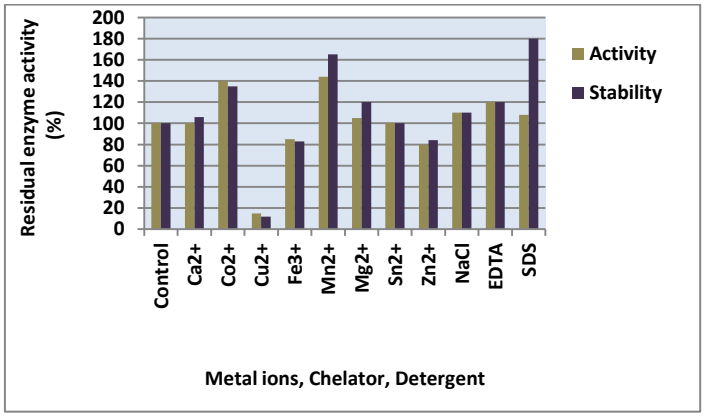
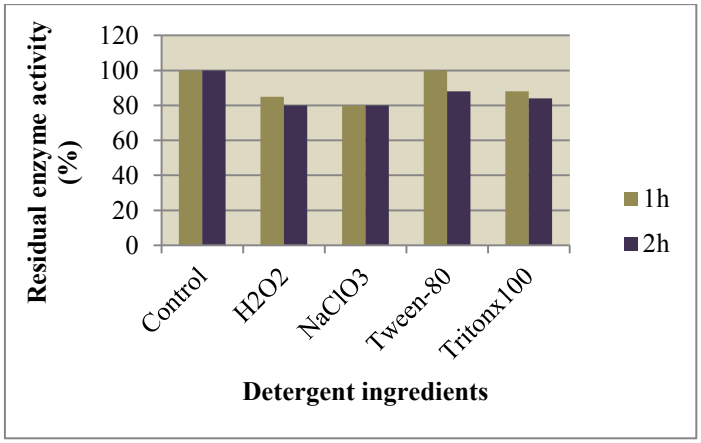
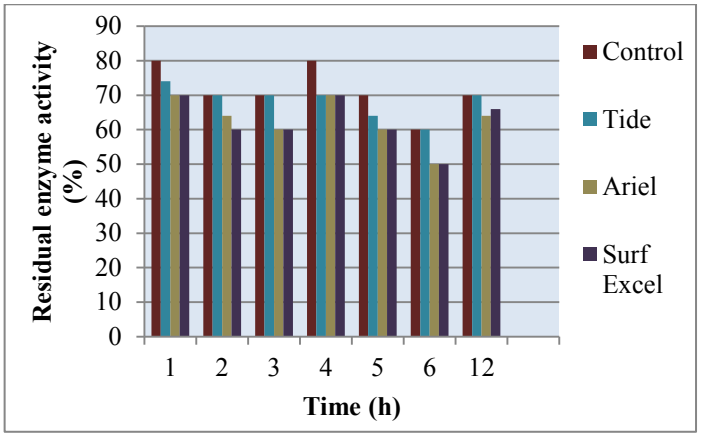


Figure 3 Effect of metal ions, chelator, salt and SDS on CB13 amylolytic activity

The activity of CB13 enzymes was found to be stimulated by salt, EDTA and SDS. SDS stimulated the activity by 80% during 1h of its incubation with the enzymes. The enzymes showed 100% to more than 80% of residual activity after 2h of incubation with selected detergent ingredients (Fig 4a). The enzymes exhibited retention of about 80% of their maximum activity till 6h of incubation in presence of various detergents (Fig 4b) as compared to control; a restoration of the activity was also seen in next 6h (Fig 4b).



(a)



(b)

Figure 4 Effect of (a) detergent ingredients (components) and (b) laundry detergents on CB13 amylolytic activity.

The CB13 amylolytic activity showed optimum activity at 40ºC at pH 11.0 which is not in agreement with the data for the amylases from *S. alluvius* ATCC 26074 (**DeMot and Verachtert, 1986**), *S. alluvius* UCD 54-83 (**DeMot and Verachtert, 1987**) *L. kononenkoae*, *C. antarctica* CBS 6678 (**DeMot and Verachtert, 1987**) and *C. flavus* (**Wanderley *et al*., 2004**). The yeast enzymes retained 45% and 98% of their maximum activity at 4ºC and 25ºC, a feature that may enable their application in various seasons and over larger geographical areas.

The enzyme is comparatively thermostable at lower temperatures, and also during boiling. It retains 20% residual activity after boiling for 30 min, a feature which is remarkable not reported so far. Amylases have earlier been reported, but they required Ca^{2+} for their stability at higher temperature e.g., *Bacillus* sp. I-3, *Bacillus* sp. ANT-6, *B. subtilis*, *Bacillus clausii* BT- 21 and *Bacillus licheniformis* (**Carvalho *et al*., 2008**). This is thus the first report of yeast amylases showing thermostability at par with the bacterial's one, but independent of the presence of Ca^{2+} for activity or stability.

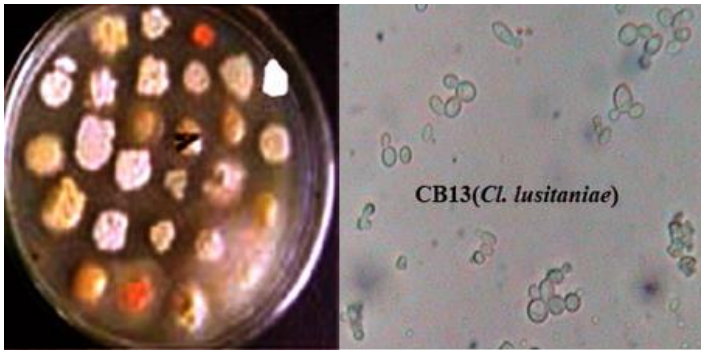
The optimal pH of the enzymes is 11.0 which is again a deviation from general acidic yeasts-amylases with optimum pH usually in the range of 4.0 and 6.0 (**Kelly *et al*., 1985; Lefuji *et al*, 1996; Wanderley *et al*., 2004**). Moreover, more than 50% of the activity of the enzymes was retained between pH 5.0 to 11.0 which is something unusual, hardly reported so far in respect of amylolytic activity from any organism. This unusual pH sturdiness is an attractive feature and forms the basis for their various industrial applications. Alkalitolerant microbes have been evaluated as important sources of various bioactive substances (**Horikoshi, 1999**), hardly a few studies have been made with respect to alkalitolerant yeasts (**Duckworth *et al*., 1996; Lisichkina *et al*., 2003**).

As opposed to earlier reports, the enzymes were inhibited by only few metal ions studied. Among inhibitors of the enzymes, Cu^{2+} and Fe^{3+} ions are general inhibitors of amylases as reported earlier (**Kelly *et al*., 1985; Aguilar *et al*., 2000; Wanderley *et al*., 2004**).Likewise, Mn^{2+} and Co^{2+} were found to be very effective activators for these enzymes as for bacterial amylases (**Bernharsdotter *et al*., 2005**). None of the metal ions tested was found to reduce the stability, rather one of them (Mn^{+2}) was found to enhance the activity during incubation with it. The enzymes were found to be moderately stimulated and stabilized by NaCl. The facts that the enzymes activity was only moderately stimulated but not stabilized by Ca^{+2} and that the activity was not affected by EDTA suggest that the enzymes are not metalloenzyme. In most of the cases, amylases have been found to be Ca^{+2} dependent metalloenzyme (**Syed *et al*., 2009**), though a few Ca^{2+} independent amylases have also been reported (**Alva *et al*., 2007**). The Ca^{2+} independent amylases are considered to be suitable for application in detergents.

The amylolytic activity was most surprisingly enhanced by SDS indicating that hydrogen bonds may not play a key role in maintaining enzyme activity (**Wang *et al*., 2005**). The enzymes were not inhibited by NaCl indicating that it is dependent on Cl^{-1}, a feature it shares with the Antarctica bacterium *P. haloplanctis* amylase and others (**Numao *et al*., 2002**). The unique characters of the enzymes were also manifested during incubation with various commercial detergents showing retention of about 80% of its maximum activity till 1h. The enzymes seem to be affected by the chemical environment of detergent to a little extent but yet, it showed much higher resistance to detergent as compared to earlier reported amylases (**Carvalho *et al*., 2008**). The biochemical properties of CB13 amylolytic activity, therefore, differ in a great deal from all the previously reported amylases (**Sivaramakrishnan *et al*., 2006**). It appears to be a cold-active nonmetallo-enzyme with a number of novel features. Preliminary biochemical characteristics suggest that these activities may find application in laundary detergent and textile industry. Since, amylases are one of the highly used enzymes in the formulation of enzymatic detergent (**Hmidet *et al*., 2009**), the enzymes may be commercially very important.

Taxonomic characterization of CB13

The yeast isolate CB13 was characterized morphologically and physiologically and assigned to the genus *Clavispora lusitaniae*. The colony was white in colour and butyrus in texture. Formations of pseudohyphae and budding cells (both unipolar and bipolar) were found. There was formation of 1-4 (typically four) ascospores per cell, each ascospore was smooth (Fig. b). The physiological data, when compared to CBS database with respect to *C. lusitaniae*, was found to be almost similar except in the utilization of starch as carbon source and Nitrate, Creatine, Creatinine and Tryptophan as nitrogen source. As against the CBS strains of *C. lusitaniae*, CB13 could utilize these metabolites as sole carbon or nitrogen source as the case may be.



(a) **(b)**

Figure 5 (a) Colonies of selected yeast-isolates including that of CB13 and (b) cells of CB13 with ascospores.

For molecular identification, the D1/D2 regions of 26s rDNA of CB13 was sequenced and the sequence was compared with those available in the NCBI database. The phylogenetic tree based on this sequence of CB13 along with those from ten the most closely related strains obtained from NCBI database showed 100 % similarity to type and many of other strains of *Clavispora lusitaniae* (Fig.6), therefore, the isolate was so named. The nucleotide sequence has been deposited in the GenBank database under Accession number JNO91166

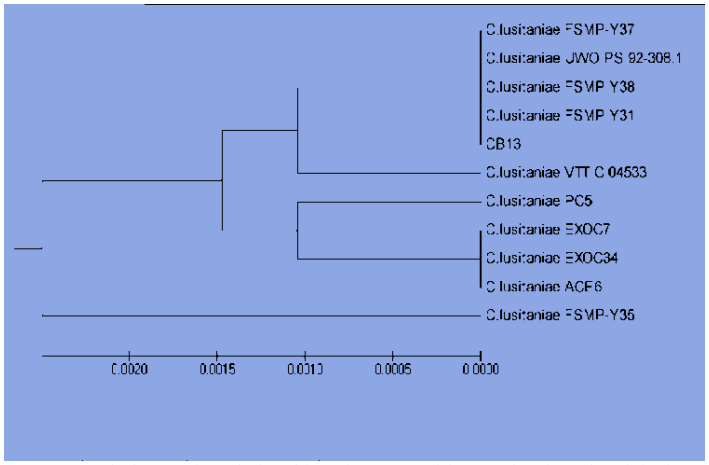


Figure 6 Phylogenetic relationship of CB13 with 10 closely related isolates retrieved from Genbank on the basis of D1/D2 sequences.

Earlier, *C. lusitaniae* has been reported to be associated with diseased lesions (**Merz *et al*., 1990; Gargeya *et al*., 1992**). Since the amylolytic activity of this strain is attractive, expression of this activity in an appropriate industrial microbe may be a safer way to exploit it (**Steven and Pretorius, 1995**). Physiological deviation in carbon and nitrogen utilization profile (Table 2) exhibited by CB13 as compared to those of other CBS strains of *C. lusitaniae* indicates the genetic flexibility of this taxon making it an adaptable organism. Moreover, this is the first report of stable, alkaline and cold-active amylolytic activity from yeast with potential application in detergent.

Reproducibility of results

All the experiments were carried out in triplicates and five times. Unless otherwise indicated, all values are average values calculated from three independently derived sets of data.

CONCLUSION

The yeast-isolates showing alkaline amylolytic activity were isolated from rotten vegetables. From among them, CB13 amylolytic activity was selected on the

basis of pH and temperature robustness for further characterization. The enzymes of CB13 were thermostable, Ca^{2+} independent, non-metalloenzyme showing compatibility with surfactants, bleaches, oxidizing agents and local powder detergents. These features suggested the CB13 enzymes to be suitable candidate for detergent preparations. The yeast-isolate CB13 was identified as *C. lusitaniae* on the basis of morphological, physiological and molecular characteristics.

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AMYLOLYTIC ACTIVITY OF KLUYVER-POSITIVE *DEBARYOMYCES OCCIDENTALIS* CELLS IMMOBILIZED IN FOAMED ALGINATE GEL

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ABSTRACT

This study investigates the amylolytic activity of Kluyver-positive yeast strain *Debaryomyces occidentalis* immobilized in foamed alginate gels. Encapsulation was performed through the traditional process of droplet formation from a foamed alginate solution. The beads were coated with a layer of 3% alginate. Amylolytic enzyme activities were determined in the presence of different carbon sources – glucose, maltose, starch or dextrin – in both complex and minimal culture media. The yeast was capable of producing inducible alpha-amylase (EC 3.2.1.1) and glucoamylase (EC 3.2.1.3). The highest level of cell proliferation was observed in the complex medium with glucose. Immobilized cells showed the highest amylolytic activities in culture media with maltose. Both alpha-amylase and glucoamylase activities were higher in minimal media than in complex media.

Keywords: *Debaryomyces occidentalis*; encapsulation; foamed alginate; Kluyver effect; alpha-amylase; glucoamylase; ethanol

INTRODUCTION

Microbial amylases began to be produced on an industrial scale during the last century. They are the most important group of enzymes, in terms of biotechnological applications. In particular, α-amylase (1,4-α-D-glucan glucanohydrolase, EC 3.2.1.1) and glucoamylase (exo-1,4-α-D-glucan glucanohydrolase, EC 3.2.1.3) have found a wide variety of uses in a number of industrial processes, including fermentation, food production and textile and paper manufacturing (**Monteiro de Souza and de Oliveira e Magalhães, 2010**). Other amylolytic enzymes can bypass the α-1,6 branch points in the starch molecule (debranching activity) (Hii et al., 2012). Amylases are produced by a wide range of organisms, including yeasts (**Yalçın and Çorbacı, 2013**). The main industrial use of amylases is in the starch saccharication process (**Hostinová and Gašperík, 2010**).

The unconventional yeast *Debaryomyces occidentalis* (syn. *Schwanniomyces occidentalis*) was identified as early as the 1980s as a 'super yeast' because of its ability to hydrolyze whole starch to glucose completely without prior hydrolysis (**Wang *et al.*, 1999**). The ability of *Debaryomyces* spp. to tolerate extreme stress could be an additionally advantage in low-cost fermentation processes (**Johnson and Echavarri-Erasun, 2011**). However, *D. occidentalis* exhibits the Kluyver effect for maltose and starch (**Weusthuis *et al.*, 1994**). Fermentation of maltose and starch is blocked under anaerobic conditions. This effect may be caused by the rapid down-regulation of sugar carrier capacity, which occurs under oxygen-limited conditions, since certain yeasts require oxygen to transport sugars into their cells and to produce extracellular amylases (**Barnett and Entian, 2005**). Kluyver effect positive yeasts play an important role in the study of immobilized cells, as they provide a specific indicator for the availability of oxygen within alginate beads.

This study is the continuation of previous research on yeast immobilization, in which *Debaryomyces* spp. cells were grown inside foamed alginate beads without undergoing irreversible structural changes to their Ca-alginate networks (**Kregiel *et al.*, 2013**). These results suggested a further avenue of enquiry, into amylolytic activity and ethanol formation in Kluyver-positive yeast cells encapsulated in foamed alginate. This manuscript describes the continuation of previous research, with a special focus on two enzymes: alpha-amylase and glucoamylase. The formation of ethanol, as the end product of yeast fermentation, is also discussed.

MATERIAL AND METHODS

Yeast strain and culture conditions

The study used amylolytic yeast *Debaryomyces occidentalis* Y500/5 from the LOCK105 Culture Collection (Lodz University of Technology, Poland). The strain was maintained on wort agar slants (MERCK-MILLIPORE) and cultured in 50 mL of complex medium [yeast extract (DIFCO) 2.5 g/L, peptone Bacto (DIFCO) 5 g/L] with 10 g/L of D-glucose (SIGMA), maltose (SIGMA), corn starch (SIGMA) or corn dextrin (SIGMA) in 500 mL round bottom flasks at 25 °C on a rotary shaker (HEIDOLPH) at 220 rpm. For the purposes of the experiment, after cultivation the cells were washed twice using Ringer solution (MERCK-MILLIPORE) with centrifugation (2000×g, 10 min, 4 oC). The concentration of yeast cells was determined using a haemocytometer, BX41 (OLYMPUS) microscope and a digital camera.

The immobilized yeast cells were cultivated in 50 mL of complex medium with a carbon source (D-glucose, maltose, corn starch or corn dextrin). The incubation was conducted on a laboratory shaker (HEIDOLPH) under aerobic conditions with constant gentle agitation (120 rpm) at 25 °C for 5 days. After 5 days of incubation inside foamed cores, the immobilized cells (100 beads) were gently washed using sterile distilled water and transferred into 100 mL of new rich medium YP [yeast extract (DIFCO) 2.5 g/L, peptone (DIFCO) 5 g/L] or fermentation performance of the immobilized cells [$(NH_4)_2SO_4$ 3 g/L; KH_2PO_4 1 g/L, $MgSO_4 \cdot 7H_2O$ 0.5 g/L, yeast extract (DIFCO) 0.5 g/L, $CaCO_3$ 3 g/L] with a carbon source (1%): glucose (G), maltose (M), natural starch from wheat (S) with 80% amylopectin and 20% amylose (MERCK-MILLIPORE) or dextrin from corn (D) (SIGMA). They were then incubated at 25 °C for 5 days on a rotary shaker (120 rpm). Samples were collected from each culture media, centrifuged (2000×g, 10 min, 4 °C) and the supernatants analyzed to determine both amylolytic activity and ethanol production.

Encapsulation

The traditional liquid-droplet-forming method was used, but from foamed alginate solution. Alginic acid salt from the brown algae was purchased from FLUKA (art. no. 71238). The specifications of this product are the most suitable for immobilization of microorganisms: molecular weight 100 000 ÷ 200 000, pH (1% in water) 6,0 ÷ 7,5 (**Blandino *et al.*, 2001**). The inoculum cells were centrifuged and suspended in 0.85% NaCl solution. To this solution was added (1:1) sodium alginate gel (4% w/w, FLUKA), which had been foamed by adding

0.1% Triton X-100 (MERCK-MILLIPORE). Triton X-100 was used as the foaming agent in a novel procedure. The toxicity of this compound was verified at the beginning of this research on foamed alginate (unpublished data). This compound at a concentration of 0.01% did not show any toxicity towards yeasts. Only chemicals of the highest purity were used. The solutions and materials were sterilized before use at 121 ºC, except for the sodium alginate solution which was sterilized using tyndallization. The mixture, which contained around 1×10^4 cells per 1 mL of gel, was dropped through a 1.8 mm needle into 0.15 M $CaCl_2$ solution. The beads (ϕ ~3 mm) were hardened in 0.15 M $CaCl_2$ solution for 60 minutes and then washed in distilled water (5 min). To study the internal morphology beads were frozen (-20ºC) and cut with a sharp razor blade. Slices of alginates were observed under a light microscope CX41 (OLYMPUS). The procedure for creating the foamed gels was described in Polish Patent No. 210458 (**Ambroziak *et al.*, 2012**). The resultant cores of foamed alginate consisted of micro-spheres approximately 50-200 microns in diameter and had a specific internal bubble structure (Fig. 1). The foamed beads were vented inside a sterile syringe under a mild vacuum at 10 ºC for 24 hours in a liquid complex medium with glucose, maltose, starch or dextrin, in order to introduce nutrients into the bead spheres. To increase mechanical stability, the foamed beads were given an additional alginate coating by placing them in a solution of 3% alginate (FLUKA) for 15 minutes. They were then washed in sterile water (**Kregiel *et al.*, 2013**).

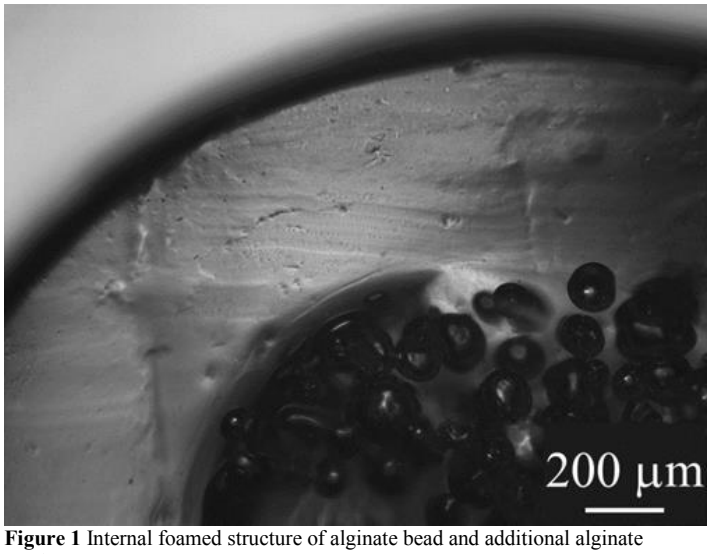

Figure 1 Internal foamed structure of alginate bead and additional alginate coating

A quantitative assessment of the immobilization was performed by dissolving the alginate beads in 0.2 M Na_2HPO_4 (in a ratio of 1 bead per 1 mL). The number of immobilized cells was determined using the microscopic method. The number of cells per single tested bead was calculated and presented as a logarithmic function.

Amylolytic and fermentation activities

Alpha-amylase activity was estimated using the 3,5-dinitrosalicylic acid method on the basis of the amount of reducing sugars released during starch hydrolysis (**Damián-Almazo *et al.*, 2008**). The reaction was carried out in 1 ml of 10 mg/mL soluble starch from corn (73% amylopectin and 27% amylose) (SIGMA) dissolved in 50mM KH_2PO_4-NaOH buffer (pH=6.0) at 45 ºC for 10 minutes. One unit of enzyme activity was defined as the amount of maltose equivalents (µmol) released per minute and per mL of supernatant (**Moranelli *et al.* 1987; Ryu and Sung, 1993**).
Glucoamylase activity was determined from the reaction of 1 mL supernatant (after cultivation in 1 ml of 10 mg/mL soluble starch from corn (SIGMA) in 50 mM citrate buffer (pH=5) at 50 ºC for 20 min. The glucose released was measured using 3,5-dinitrosalicylic acid reagent with glucose as a standard. One glucoamylase activity unit (U) was defined as the amount of enzyme that releases one µmol of glucose equivalent per minute per mL (**Clementi and Rossi 1986; Ryu and Sung, 1993**).
The fermentation performance of the immobilized cells was evaluated based on the ethanol content in the precipitate, determined using an AGILENT 6890 gas chromatograph equipped with headspace autosampler, capillary INNOVAX column (60 m × 0.32 mm) and flame ionization detector (FID) (**Kregiel *et al.*, 2012**).

The experiments were carried out in triplicate, and the standard deviation was calculated.

RESULTS AND DISCUSSION

Yeast cell growth inside gel beads

After a 5-day incubation period in complex medium with glucose YPD, the number of immobilized yeast cells increased from an initial 2×10^4 to 1.2×10^7 per bead, indicating significant colonization of the alginate cores (Fig. 2). Multiplication inside beads incubated in complex media with other carbon sources was slightly lower at $5\div6\times10^6$ cells/bead.

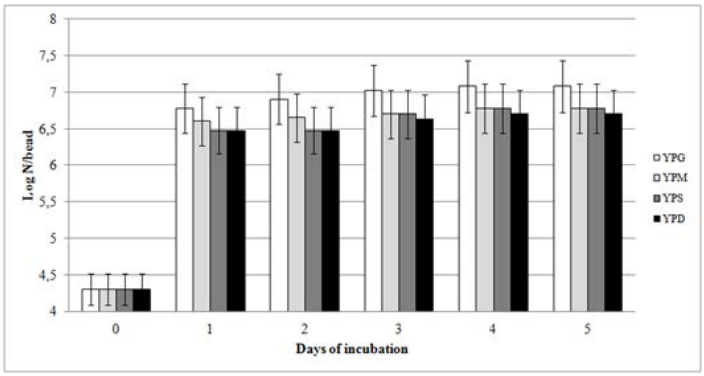

Figure 2 Growth of *Debaryomyces occidentalis* in foamed alginate beads

Oxygen may influence the production of glycolytic enzymes (**Fukuhara, 2003**). **Violle *et al.*** (1992) have shown that amylase and glucosidase are not synthetized in the presence of maltose under anaerobic conditions. However, maltose permease is synthetized in anaerobiosis without its functioning being inhibite. In our studies, foamed alginate cores with special interior spaces provided favorable conditions for the synthesis of amylolytic enzymes.
The alpha-amylase activity in rich culture media gradually increased, reaching the highest value (2.65 U/mL) in YPM medium with maltose as the carbon source (Fig. 3A). No such gradual increase in alpha-amylase activity was observed during the cultivation of immobilized yeast cells in minimal medium Mo, although the highest value of alpha-amylase activity was again in the medium with maltose as the carbon source (3.9 U/mL) (Fig. 3B).

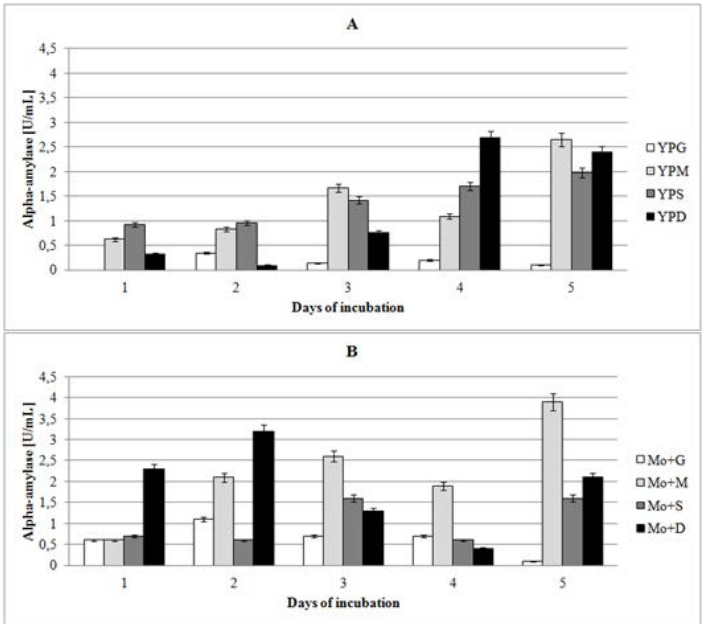

Figure 3 Alpha-amylase activity in A) complex medium, B) minimal medium

The maximal activities of glucoamylase were also noted in culture media with maltose, at 0.08 U/mL and 0.11 U/mL for rich and minimal medium, respectively (Fig. 4 AB).

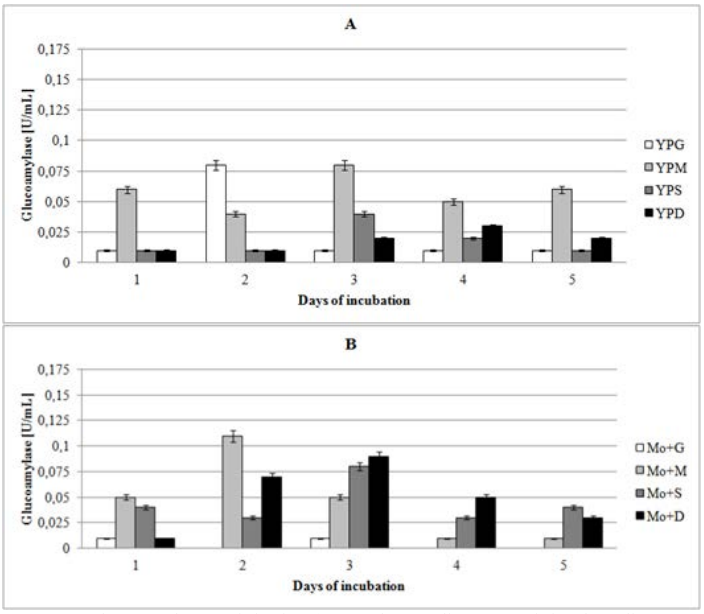

Figure 4 Glucoamylase activity in A) complex medium, B) minimal medium

These results confirm that amylases show substrate specificity, and that the production of amylolytic enzymes is subject to catabolite repression by glucose (Gupta et al., 2003).

Amylolytic activity was detected in the culture media throughout the cultivation period, except in the media with glucose as a carbon source. This may be due to the high stability of these enzymes. **Flores-Maltos *et al.* (2011)** have found that cross-linking the alginate with divalent ions (such as Ca^{2}+) improves the stability of enzymes. Figure 5 shows that immobilized *D. occidentalis* produced ethanol with different carbon sources.

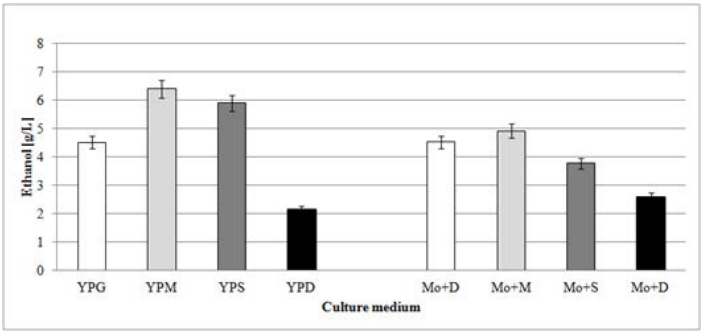

Figure 5 Ethanol formation by encapsulated yeast

The highest levels of ethanol production were obtained in YPM and Mo medium with maltose, at 6.4 and 4.9 g/L, respectively. These results indicate that in the foamed alginate cores with Kluyver-positive cells, takes place not only the synthesis of amylolytic enzymes, but also alcohol fermentation in one-step process. These and previously reported data (**Kregiel *et al.*, 2013**) shows that yeast encapsulation in foamed alginate seems to have an excellent potential for inexpensive, low energy and repeatable process of starch hydrolysis.

CONCLUSION

The method of cell encapsulation in foamed alginate presented in this paper allows not only for good growth of Kluyver-positive yeast within the bead spaces, but also for the production of amylases, using various carbon sources. This immobilization technique may be an effective way to produce stable amylolytic enzymes, which are of great scientific interest and have a broad range of industrial applications.

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BIODIVERSITY OF MYCOBIOTA IN PEANUT SEEDS, CORN AND WHEAT GRAINS WITH SPECIAL REFERENCE TO THEIR AFLATOXIGENIC ABILITY

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ABSTRACT

The fungal biodiversity in 30 samples, 10 of each of peanut seeds, corn and wheat grains were estimated on two isolation media (AFPA and DRBC). The aflatoxigenic ability of isolates related to *Aspergillus* section *Flavi* using CAM agar plates was also assessed. The results indicated that the mean moisture content was relatively low in peanut (8.05%) while it was relatively high in corn samples (10.45%). A limited number of peanut seeds but a large number of corn grains were fungi-free on both media. On AFPA, the aflatoxigenic species contaminated 9, 5 and 7 of peanut, corn and wheat samples respectively. On AFPA and DRBC, the total number of genera and species recorded on wheat were higher than those on corn and peanut and only three genera, *Aspergillus*, *Penicillium* and *Fusarium* were isolated from the three substrates. *Aspergillus* possessed more propagules on peanut than on corn and wheat, whereas *Fusarium* and *Penicillium* had more propagules on corn than on peanut and wheat. The aflatoxigenic species *A. flavus*, and *A. niger* were isolated from the three substrates. These two species showed higher propagules on peanut and corn than on wheat. *P. chrysogenum*, *P. duclauxii* and *F. verticillioides* were recorded from corn and wheat grains, *P. aurantiogriseum* and *F. chalamydosporum* from corn and *P. brevicompactum, P. funiculosum* from wheat and *P. pinophilum* and *F. oxysporum* from peanut only. Flourescence at 365 nm of 43 *A. flavus* and other 4 fungal strains recovered from the analyzed substrates and grown on CAM agar plates revealed that all *A. flavus* strains showed blue color with different intensities indicating aflatoxin B production, while the other 4 non-*A. flavus* strains showed negative results.

Keywords: Peanut, corn, wheat, aflatoxigenic fungi, aflatoxins

INTRODUCTION

Most agricultural commodities are susceptible to fungal invasion before, during or after harvesting, owing to improper handling, drying, transportation and/or storage. Food contamination by fungi and their toxic metabolites (mycotoxins) still remains a serious global problem. It is estimated that as much as 25 % of the worlds' cereals are contaminated with fungi and known mycotoxins, while a higher percentage could be contaminated with toxins are yet unidentified (**Mannon and Johnson, 1985**). Peanuts and cereals are foods of intermediate moisture content, thus fungal and mycotoxin development may occur at the farm or at the site of storage, affects the yield, quality and nutritive value of the products. The degree of mould contamination in stored grains can be used as a measure of their quality (**Karunaratne and Bullerman, 1990**). Moreover, some grains may contain mycotoxins as a result of fungal growth (**CAST, 1989; FAO, 1990**). Mycotoxins which occur naturally in foods are of significance in terms of food safety. These are produced mainly by species of *Aspergillus*, *Penicillium* and *Fusarium* (**Bullerman, 1979**).

In countries like Egypt, where a hot and humid climate prevails, agricultural practices favour mould growth (**Rustom, 1997**), data on moulds, their mycotoxins as well as other deteriorating factors in foods are urgently needed. Peanuts, corn and wheat grains, which contribute protein and calories, form the basis for the staple diet of the majority of the people in Egypt and other developing countries. In general, such seeds and grains are high risk crops (**Mirocha, 1983**). Historically only *A. flavus*, *A. parasiticus* and *A. nomius* have been known as producers of aflatoxins (**Ehrlich *et al.*, 2007**). However, **Coppock and Christian (2007)** have since reported that *A. bombycis*, *A. ochraceoroseus* and *A. pseudotamarii* are also capable of producing aflatoxins.

The objective of this study was to investigate the mycobiota and possibly the aflatoxigenic species associated with peanut seeds, corn and wheat grains grown in Egypt.

MATERIAL AND METHODS

Collection of peanut, corn and wheat samples

Thirty samples, 10 of each of peanut seeds, corn and wheat grains were collected from Assiut and Sohag Governorates after harvesting during the period from January to May 2013. These samples were from those marketed for human consumption and were apparently in good conditions. Each sample (1 Kg each) was placed in additional sterile polyethylene bag. The samples were transferred into the laboratory and kept at 4ºC pending moisture content determination and mycological analyses.

Determination of moisture content

The moisture content was estimated by drying triplicates of known weight of the samples (peanut, corn and wheat) at 110º C for 24 h and then reweighed (**Magan and Lacey, 1985; Pitt and Hocking, 2009**). The moisture content is expressed as the average percentage of the weight loss of the three replicates.

Isolation, enumeration and identification of fungi

Two different media were used to assess the mycological quality of the samples: one for general isolation of fungi (dichloran rose Bengal chloramphenicol agar, DRBC of **King *et al.*, 1979,** modified by **Pitt and Hocking, 1997**), and the other for selective isolation of *Aspergillus flavus* and *Aspergillus parasiticus* fungi (*Aspergillus flavus/parasiticus* agar, AFPA of **Pitt *et al.*, 1983**).

Direct plating technique adopted by the second international workshop (**Pitt *et al.*, 1992**) was used, as it was recommended for isolating fungi from peanut seeds. The seeds and grains were briefly surface disinfected by 50 ml house-hold chlorine bleach (nominally 4-5 % active chlorine) added to 450 ml distilled water

for 2 minutes before culture. Peanut seeds, corn and wheat grains were cultured on the surface of agar plates (5 plates/sample, 5 particles/plate, with a total of 25 seeds or grains on each medium type for each sample).
DRBC agar plates were incubated at 28ºC for 7–10 days after which the growing colonies were counted, identified whenever possible from the original plates, otherwise sub-cultured on potato dextrose agar (PDA) slants, allowed to grow, then stored at 4ºC for later identification. On the other hand, the inoculated plates of AFPA medium were incubated for 42-48 hours at 30°C. After incubation the reverse of the Petri-dishes was examined for a bright yellow/orange coloration (**Pitt *et al.*, 1983; Pitt and Hocking, 2009**). The identification of the aflatoxigenic aspergilli and other moulds was confirmed on the basis of their macroscopic and microscopic features.

Identification of fungi

The identification of fungi was carried out using the methods, media and plating techniques described by **Raper and Fennell (1965); Ellis (1971); Pitt (1979); Moubasher (1993); Leslie and Summerell (2006); Domsch *et al.* (2007); and Pitt and Hocking (2009).**

Screening for aflatoxins

All *Aspergillus flavus* strains (43) in addition to some other fungal strains (4) collected during this study were screened for aflatoxin-producing ability using coconut agar medium (CAM). The medium was prepared according to **Davis *et al.* (1987)** as follows: 100 g of shredded coconut was homogenized for 5 min with 300 ml hot distilled water, then the homogenate was filtered through four layers of cheese cloth, completed to 1000 ml by distilled water, the pH of the clear filtrate was adjusted to pH 7 and 20 g agar were added. The medium was autoclaved for 15 min at 121ºC, cooled to about 40º – 45ºC, and poured while being stirred into sterile Petri-dishes. Fungal isolates were inoculated at the center of CAM agar plates and incubated at 25ºC in the dark for 7 days. Cultures were observed for flourescence under long-wave UV light (365 nm) after 3, 5 and 7 days. The positive results were shown as blue fluorescence and an uninoculated plate was observed as a reference.

RESULTS AND DISCUSSION

Moisture content of the samples

The moisture content in peanut samples ranged from 5.94 to 10.27% with a mean of 8.05±1.25 while those in corn grain samples fluctuated from 9.29 to 13.40% with a mean of 10.45±1.18. In wheat grain samples they varied from 7.51% to 11.58% with a mean of 9.56±1.24 (Table 1). In this respect, the moisture content of peanut seed samples collected from Uganda and Kenya ranged from 5.07% to 7.97% with a mean of 6.63% (**Ismail, 2000**), while for those collected from Kenya ranged from 3.3% to 6.9% (**Wagacha *et al.*, 2013**), and of maize collected from Egypt ranged from 8.75-16.76% (**Abdel-Hafez *et al.*, 2014**).

Overview on the mycobiota of peanut seeds and corn and wheat grains

From the summarized data presented in table (1) it could be noted that all samples from peanut seeds and wheat grains collected from Assiut and Sohag Governorates were infested with fungi as revealed on AFPA and DRBC media. However only 9 out of 10 samples of corn were contaminated as revealed on both media.
On AFPA, the aflatoxigenic species, that showed orange brown pigmentation after 48 hours of incubation at 30ºC (Fig 1), contaminated a total of 9, 5 and 7 of peanut, corn and wheat samples respectively. In this respect all 144 *A. flavus* isolates isolated from Algerian wheat were of bright orange reverse on AFPA plates (**Riba *et al.*, 2010**).
On DRBC, 10, 8 and 5 samples yielded aflatoxigenic species of the analyzed substrates. From peanut seeds analyzed from both governorates, 94.8% and 99.6% were infested with fungi on AFPA and DRBC, respectively, while only 42.8% and 47.2% of corn grains were contaminated with fungi on the two isolation media, respectively. Regarding wheat samples only 55.2% and 64% of the grains were contaminated with fungi on both media respectively (Table 1).
Limited numbers of peanut seeds (13 out of 250 on AFPA and only 1 on DRBC), while large numbers of corn grains (143 out of 250 and 132 out of 250) were fungi-free on AFPA and DRBC, respectively. On the other hand, 112 out of 250 and 90 out of 250 wheat grains were fungi-free on both media respectively. Regarding fungal isolates from the three substrates (peanut, corn and wheat), the largest number was reported from peanut (403 and 467/250 seeds analysed) while the least numbers were reported from corn (119 and 126/250 grains) on AFPA and DRBC, respectively (Table 1).

Fungal diversity in peanut seeds and corn and wheat grains on *Aspergillus flavus/parasiticus* agar medium (AFPA)

All peanut and wheat samples but nine corn samples were contaminated with fungi. However more propagules were obtained from peanut. The total number of genera and species recorded on wheat (13 genera and 20 species) were higher than those obtained on corn (8 and 17) and peanut (4 and 5) (Table 2). Only two genera, *Aspergillus* and *Penicillium* were isolated from the three substrates investigated (peanut, corn and wheat) on AFPA at 30ºC. *Aspergillus* possessed more propagules on peanut than on corn and wheat, but *Penicillium* had more propagules on corn than on peanut and wheat. From *Aspergillus*, the aflatoxigenic species (*Aspergillus flavus*), and *A. niger* were recovered from the three substrates. Both species showed high propagules on peanut and corn than on wheat (Table 2). These two species were also the most dominant in maize from Egypt (**Abdel-Hafez *et al.*, 2014**). In addition, two more *Aspergillus* species were recovered from corn (*A. fumigatus* and *A. tamarii*) but not from peanut or wheat, and two species were recovered from wheat (*A. candidus* and *A. clavatonanica*) but not from peanut or corn (Table 2). In this respect, **Riba *et al.* (2010)** found that out of the 150 strains of *Aspergillus* section *Flavi* isolated from Algerian wheat, 144 were identified as *Aspergillus flavus* and 6 as *Aspergillus tamarii*. Other *Aspergillus* species isolated belonged to the section *Nigri*, *Circumdati* and *Terrei*. **Abriba *et al.* (2013)** registered *Aspergillus flavus, A. parasiticus, A. terreus, A. niger, A. versicolor, A. ochraceus* and *A. nidulans* from peanut, maize and/or wheat in Nigeria.
None of *Penicillium* species was recorded from the three substrates investigated. *P. chrysogenum* was isolated from both corn and wheat grain samples only, *P. aurantiogriseum* and *P. duclauxii* from only corn and *P. brevicompactum* and *P. funiculosum* from only wheat. Unidentified *Penicillium* species was recorded from peanut and corn. Unidentified species of *Penicillium* were recorded from peanut, maize and/or wheat in Nigeria (**Abriba *et al.*, 2013**) and from maize in Egypt (**Abdel-Hafez *et al.*, 2014**).
Rhizopus stolonifer was recorded in high frequency from peanut and in rare frequency from wheat but was missing from corn. In agreement with our findings, species of *Rhizopus* were reported as dominant from peanut in Uganda (**Ismail, 2000**), but missing in maize from Egypt (**Nooh *et al.*, 2014**), however they were reported from maize and wheat but not from peanut in Nigeria (**Abriba *et al.*, 2013**).
Acremonium (2 species), *Chaetomium globosum*, *Nigrospora oryzae*, *Rhizoctonia solani* and species of *Fusarium* were recorded from corn and wheat but not from peanut. Also, *Acremonium potronii, Fusarium nygamai, F. oxysporum*, *F. verticillioides* were recorded only from corn but *Acremonium strictum* from only wheat. Some more species were registeded only from wheat grains (*Alternaria* represented by *A. alternata, A. chlamydospora* and *Alternaria* sp., *Epicoccum nigrum, Phaeoacremonium* sp., *Scytalidium lignicola* and *Stemphylium botryosum*, all of them are dematiaceous hyphomycetes), but *Mucor* and sterile mycelia from only peanut and *Trichothecium roseum* from only corn. Species of *Fusarium*, *Alternaria*, *Botryotrichum*, *Cladosporium*, *Setosphaeria* and *Tichothecium* were also reported from Egyptian maize (**Abdel-Hafez *et al.*, 2014**).

Figure 1 Bright yellow/orange coloration on AFPA indicting aflatoxigenic *Aspergillus* species

Table 1 Summarized data for the mycobiota analysis of peanut, corn and wheat samples collected from Assiut (A) and Sohag (S) Governorates on AFPA and DRBC media

Substrate	Peanut						Corn						Wheat					
Medium	AFPA			DRBC			AFPA			DRBC			AFPA			DRBC		
Governorate	A	S	Total	A	S	Total	A	S	Total	A	S	Total	A	S	Total	A	S	Total
Samples analyzed	5	5	10	5	5	10	5	5	10	5	5	10	5	5	10	5	5	10
Samples infested with fungi	5	5	10	5	5	10	4	5	9	4	5	9	5	5	10	5	5	10
Samples infested with aflatoxigenic species	4	5	9	5	5	10	3	2	5	4	4	8	4	3	7	2	3	5
Seeds (grains) analyzed	125	125	250	125	125	250	125	125	250	125	125	250	125	125	250	125	125	250
Seeds (grains) infested with fungi	125	112	237	125	124	249	40	67	107	38	80	118	60	78	138	57	103	160
% seeds (grains) infested with fungi	100	89.6	94.8	100	99.2	99.6	32	53.6	42.8	30.4	64	47.2	48	62.4	55.2	45.6	82.4	64
Fungi-free seeds (grains)	0	13	13	0	1	1	85	58	143	87	45	132	65	47	112	68	22	90
% fungi-free seeds (grains)	0	10.4	5.2	0	0.8	0.4	68	46.4	57.2	69.6	36	52.8	52	37.6	44.8	54.4	17.6	36
Total isolates obtained	250	153	403	241	226	467	49	70	119	43	83	126	62	78	140	64	112	176
Total aflatoxigenic isolates obtained	97	29	126	71	34	105	17	5	22	12	7	19	10	6	16	3	4	7
Genera	2	4	4	5	7	8	4	8	8	4	7	7	8	9	13	11	10	13
Species	3	5	5	9	9	13	6	16	17	6	11	14	14	10	20	16	11	22
% moisture contents: mean ± SD [min-max]	8.1±1.3 [5.94-10.27]						10.5±1.2 [9.29-13.40]						9.56±1.2 [7.51-11.58]					

Table 2 Colony forming units (CFUs, calculated per 250 seeds or grains in all samples), percentage CFUs and frequency of fungi isolated from peanut seeds, corn and wheat grains recovered on *Aspergillus flavus/parasiticus* agar at 30ºC.

Fungi	Peanut			Corn			Wheat		
	CFUs	%CFUs	%F	CFUs	%CFUs	%F	CFUs	%CFUs	%F
Acremonium				2	1.68	10	1	0.71	10
A. potronii Vuillemin				2	1.68	10			
A. strictum W. Gams							1	0.71	10
Alternaria							59	42.14	100
A. alternata (Fries) Keissler							52	37.14	90
A. chlamydospora Mouchacca							2	1.43	10
Alternaria sp.							5	3.57	30
Aspergillus	228	56.58	100	59	49.58	70	25	17.86	80
A. candidus Link							1	0.71	10
A. clavatonanica Bat. *et al.*							2	1.43	10
A. flavus Link	126	31.27	90	22	18.49	50	16	11.43	70
A. fumigatus Fresenius				1	0.84	10			
A. niger van Tieghem	102	25.31	90	35	29.41	70	6	4.29	40
A. tamarii Kita				1	0.84	10			
Chaetomium globosum Kunze				1	0.84	10	5	3.57	30
Epicoccum nigrum Link							3	2.14	30
Fusarium				12	10.08	60	4	2.86	20
F. nygamai Burgess & Trimboli				2	1.68	20			
F. oxysporum Schlechtendal				2	1.68	20			
F. verticillioides (Saccardo) Nirenberg				6	5.04	30			
Fusarium sp.				2	1.68	20	4	2.86	20
Mucor sp.	1	0.25	10						
Nigrospora oryzae (Berkeley & Broome) Petch				4	3.36	20	13	9.29	40
Penicillium	7	1.74	40	35	29.41	70	6	4.29	40
P. aurantiogriseum Dierckx				6	5.04	40			
P. brevicompactum Dierckx							1	0.71	10
P. chrysogenum Thom				11	9.24	30	4	2.86	20
P. duclauxii Delacroix				14	11.76	70			
P. funiculosum Thom							1	0.71	10
Penicillium sp.	7	1.74	40	4	3.36	10			
Phaeoacremonium sp.							1	0.71	10
Rhizoctonia solani Kühn				5	4.20	30	1	0.71	10
Rhizopus stolonifer (Ehrenberg) Vuillemin	151	37.47	100				14	10	10
Scytalidium lignicola Pesante							1	0.72	10
Stemphylium botryosum Wallr.							7	5	20
Sterile mycelia (dark & white)	16	3.97	60						
Trichothecium roseum (Persoon: Fries) Link				1	0.84	10			
Total number of propagules	403	100	100	119	100	90	140	100	100
Number of genera		4			8			13	
Number of species		5			17			20	

Fungal diversity in peanut seeds and corn and wheat grains on dichloran rose Bengal chloramphenicol agar medium (DRBC)

As the case on AFPA, more propagules were obtained from peanut than from wheat or corn on DRBC medium. On the other hand, broader spectrum of species was recorded on wheat (22 species) than on corn (14) or peanut (13) (Table 3).
Three genera, *Aspergillus, Fusarium* and *Penicillium* were isolated from the three substrates (Table 3).
Aspergillus possessed more propagules on peanut than on corn and wheat, while *Fusarium* and *Penicillium* had more propagules on corn than on peanut and wheat. From *Aspergillus*, the aflatoxigenic species (*Aspergillus flavus*) and *A. niger* were recovered from the three substrates. Both species showed higher propagules on peanut and corn than on wheat (Table 3). Several studies reported the predominance of *A. flavus* in peanut samples originating from Egypt (**El-Shanshoury *et al.*, 2014**), Nigeria (**Abriba *et al.*, 2013**), Uganda (**Ismail, 2000**), Kenya (**Wagacha *et al.*, 2013**), Iran (**Hedayati *et al.*, 2010**), Libya (**Attiatalla *et al.*, 2010**) and Saudi Arabia (**Deabes and Al-Habib, 2011**), in maize samples originating from Egypt (**Nooh *et al.*, 2014; Abdel-Hafez *et al.*, 2014**), Kenya (**Muthomi *et al.*, 2012**), Malaysia (**Reddy and Salleh, 2011**), Nigeria (**Ezekiel *et al.*, 2012**), Hungary (**Toth *et al.*, 2012**), and in wheat grain samples originating from Egypt (**Mazen *et al.*, 1984; Abdel-Hafez *et al.*, 1990; El-Shanshoury *et al.*, 2014**), Australia (**Berghofer *et al.*, 2003**), Argentina (**Vaamonde *et al.*, 2003**), Iran (**Ghiasian et al., 2004**), Nigeria (**Abriba *et al.*, 2013**), Libya (**Attitalla *et al.*, 2010**) and Turkey (**Bayder *et al.*, 2005**). It was also found that

the incidence of aflatoxigenic species *A. flavus* was higher in peanuts (69 %, 49. 38 %) than in wheat (13 %, 7.67 %) in Argentina (**Vaamonde *et al.*, 2003**) and Libya (**Attitalla *et al.*, 2010**), respectively.

In addition only one more *Aspergillus* species was recovered on peanut and wheat (*A. candidus*) but not from corn, while *A. terreus* was recovered on peanut and corn but not from wheat, and *A. fumigatus* was recovered on wheat but not from peanut or corn (Table 3). These aspergilli and others were previously reported from peanut, maize and/or wheat (*A. niger*; **Reddy and Salleh, 2011; El-Shanshoury *et al.*, 2014**), maize in Egypt (*A. terreus*; **El-Shanshoury *et al.*, 2014**), maize grains in Kenya (*A. flavus, A. fumigatus, A. niger, A. ochraceus, A. terreus, A. versicolor* and *A. clavatus*; **Muthomi *et al.*, 2012**).

None of *Fusarium* species was recorded from the three investigated substrates. Only *F. verticillioides* and an unidentified *Fusarium* species were recovered from both corn and wheat, *F. chlamydosporum* from corn, and *F. oxysporum* from peanut. Also, none of *Penicillium* species was represented in the three substrates. Only *P. chrysogenum* and *P. duclauxii* were recorded from both corn and wheat, *P. funiculosum* and *P. pinophilum* from peanut, *P. aurantiogriseum* from corn, and an unidentified *Penicillium* species was recorded from peanut and corn (Table 3). *Fusarium verticillioides, F. graminearum, F. proliferatum, F. equiseti* and *Penicillium* sp. were prevalent in corn samples in Malaysia (**Reddy and Salleh, 2011**), while the most dominant *Fusarium* species in maize grains in Egypt were in the following order: *F. verticillioides*, *F. oxysporum*, *F. solani*, *F. proliferatum*, *F. udum* and *F. nisikadoi* (**Abdel-Hafez *et al.*, 2014**).

Some other species were isolated from two substrates but not from the third e.g. *Nigrospora oryzae* from corn and wheat but not from peanut, *Rhizopus stolonifer* in high frequency from peanut and in low frequency from wheat but not from corn, and *Rhizoctonia solani* from peanut and corn but not from wheat. On the other hand, some other species were recorded from one substrate but not from the others e.g. *Macrophomina phaseolina*, unidentified species of *Acremonium* and *Trichoderma* and yellow sterile mycelia were recorded from peanut; *Alternaria alternata*, *Alternaria* sp., *Botroytrichum piluliferum, Chaetomium elatum, C. globosum*, *Chaetomium* sp., *Cladosporium cladosporioides, C. oxysporum, Epicoccum nigrum, Eurotium amstelodami, Stemphylium botryosum* and *Thermoascus aurantiacus* were recorded from only wheat; and *Chaetomium spirale* and *Setosphaeria rostrata* were recorded from only corn (Table 3). In this respect, six fungal genera, namely *Aspergillus, Penicillium,* and *Fusarium* from peanut, maize and wheat, and *Mucor* (maize), *Rhizopus*, *Alternaria* and *Cladosporium* (wheat) (**El-Shanshoury *et al.*, 2014**) and from Egyptian maize samples collected from 10 governorates including Assiut and Sohag prior to storage, however *Alternaria* and *Rhizopus* were not recorded from samples collected from Assiut and Sohag ((**Nooh *et al.*, 2014**). Also species of *Mucor* and *Rhizopus* dominated some corn samples collected from Malaysia (**Reddy and salleh, 2011**). **Misra *et al.* (2010)** isolated 16 co-existing fungal species with *A. flavus* as frequent on wheat grains in India and these were *A. fumigatus, A. japonicus, A. niger, A. tamarii, Emericella nidulans, Candida albicans*, *Chaetomium globosum, C. spirale, Cladosporium cladosporioides, Fusarium oxysporum, Memnoniella echinata, Mucor hiemalis, Penicillium citrinum, P. funiculosum, Torula convoluta* and *Trichoderma viride*. **Ismail (2000)** found also that the most common fungal species in peanuts collected from Kenya and Uganda were *Rhizopus stolonifer*, *Aspergillus parasiticus*, *Fusarium solani*, *Lasiodiplodia theobromae* and *Penicillium chrysogenum* on DRBC. **Abriba *et al.* (2013)** recorded species of *Aspergillus* (*A. flavus, A. parasiticus, A. terreus, A. niger, A. versicolor, A. ochraceus, A. nidulans*), *Penicillium* (2 species) and *Rhizopus* (1 species) from peanut, maize and/or wheat in Nigeria.

Flourescence of aflatoxigenic species and other fungal strains recovered from peanut seeds, corn and wheat grains on coconut agar medium (CAM)

The fluorescence at 365 nm of 47 fungal strains grown on CAM was observed. Twelve out of the 15 *Aspergillus flavus* strains recovered from peanut seeds showed intense blue color indicating aflatoxin B production and only 3 weak aflatoxigenic strains (showing faint colour), one of them (AUMC 9769) fluoresced greenish yellow color indicating aflatoxin G production (Fig. 2). The other 4 strains originating from peanut seeds (related to *Aspergillus candidus, A. niger, Macrophomina phaseolina* and *Penicillium funiculosum*) showed negative results (Table 4). Out of 11 *Aspergillus flavus* strains recovered from corn grain samples, only two showed strong aflatoxigenic ability (intense blue color) while 9 strains showed faint blue color indicating low aflatoxin-producing ability (Table 4). The flourescence at 365 nm of 17 *A. flavus* strains recovered from wheat grains revealed 5 strains as very highly aflatoxigenic and 5 strains were also highly aflatoxigenic (intense blue color), but only 7 strains showed faint blue color indicating lower production of aflatoxin B (Table 4). In this respect, **Riba *et al.* (2012)** screened 150 isolates that belong to *A. flavus* (144 isolates) and *A. tamarii* (6) isolated from Algerian wheat for aflatoxin production on coconut agar medium (CAM), and found only 45 isolates (30 %) were aflatoxigenic. Also, in the screening of **Ezekiel *et al.* (2012)** of 90 isolates of *Aspergillus* section *Flavi* on neutral red desiccated coconut agar medium (NRDCA) it was found that only 35.6 % of the isolates produced the characteristic fluorescence of aflatoxins.

Table 3 Colony forming units (CFUs, calculated per 250 seeds or grains in all samples), percentage CFUs and frequency of fungi isolated from peanut seeds, corn and wheat gra recovered on dichloran rose Bengal chloramphenicol agar at 28ºC.

Fungi	Peanut			Corn			Wheat		
	CFUs	CFUs%	F%	CFUs	CFUs%	%F	CFUs	CFUs%	%F
Acremonium sp.	1	0.21	10						
Alternaria							70	39.77	100
A. alternata							69	39.20	100
Alternaria sp.							1	0.57	10
Aspergillus	284	60.81	100	38	30.16	80	13	7.38	70
A. candidus	1	0.21	10				4	2.27	10
A. flavus	105	22.48	100	19	15.08	80	7	3.98	50
A. fumigatus							1	0.57	10
A. niger	177	37.90	100	18	14.29	70	1	0.57	10
A. terreus Thom	1	0.21	10	1	0.79	10			
Botrytrichum piluliferum Saccardo & Marchal							1	0.57	10
Chaetomium				4	3.17	30	13	7.38	30
C. elatum Kunze							2	1.14	10
C. globosum							7	3.98	30
C. spirale Zopf				4	3.17	30			
Chaetomium sp.							4	2.27	10
Cladosporium							12	6.82	60
C. cladosporioides (Fres.) de Vries							10	5.68	40
C. oxysporum Berk. & M.A. Curtis							2	1.14	20
Epicoccum nigrum							5	2.84	50
Eurotium amstelodami Mangin							1	0.57	10
Fusarium	6	1.28	30	14	11.11	70	5	2.84	20
F. chalamydosporum Wollenweber & Reinking				8	6.35	30			
F. oxysporum	6	1.28	30						
F. verticillioides				3	2.38	20	4	2.27	10
Fusarium sp.				3	2.38	20	1	0.57	10
Macrophomina phaseolina (Tassi) Goidanch	8	1.71	40						
Nigrospora oryzae				11	8.73	20	7	3.98	50
Penicillium	40	8.57	40	52	41.27	90	3	1.70	20
P. aurantiogriseum				1	0.79	10			
P. chrysogenum				13	10.32	30	1	0.57	10
P. duclauxii				28	22.22	40	2	1.14	10
P. funiculosum	1	0.21	10						

P. pinophilum Hedgcock	12	2.57	20						
Penicillium sp.	27	5.78	40	10	7.93	40			
Rhizoctonia solani	3	0.64	30	2	1.59	10			
Rhizopus stolonifer	122	26.12	90				19	10.80	30
Setosphaeria rostrata Leonard				5	3.97	10			
Stemphylium botryosum							26	14.77	50
Sterile mycelia (yellow)	1	0.21	10						
Thermoascus aurantiacus Miehe							1	0.57	10
Trichoderma sp.	2	0.43	20						
Total number of propagules	467	100	100	126	100	90	176	100	100
Number of genera		8			7			13	
Number of species		13			14			22	

Table 4 Flourescence (at 365 nm) of *Aspergillus flavus* and other fungal strains recovered from peanut seeds, corn and wheat grains as revealed on coconut agar medium (CAM).

Substrate	**Peanut**		**Corn**		**Wheat**	
Species	AUMC No.	Flourscence on CAM	AUMC No.	Flourscence on CAM	AUMC No.	Flourscence on CAM
A. flavus	9768	++	9783	++	9806	+++
A. flavus	9769	+ GY*	9784	+	9808	+
A. flavus	9770	++	9785	+	9810	+++
A. flavus	9771	+	9786	+	9813	++
A. flavus	10135	++	9787	+	10133	+
A. flavus	9801	++	9788	+	9816	++
A. flavus	9772	++	9790	++	9817	+
A. flavus	9773	++	9793	+	9843	++
A. flavus	9778	++	9794	+	9848	+
A. flavus	9779	++	9796	+	9849	+++
A. flavus	9780	++	9797	+	9850	+
A. flavus	10134	+	--		9851	++
A. flavus	9781	++	--		9852	+++
A. flavus	9782	++	--		9853	+++
A. flavus	9803	++	--		9854	+
A. flavus	--		--		9855	+
A. flavus	--		--		9856	++
A.candidus	9777	-ve	--		--	
A. niger	9802	-ve	--		--	
Macrophomina phaseolina	10137	-ve	--		--	
Penicillium funiculosum	9776	-ve	--		--	
Total *flavus*-strains tested	15		11		17	
Total positive strains	15		11		17	
Total negative strains	4 (non-*flavus*)		0		0	

*GY = Greenish yellow fluorescence, fluorescence on CAM is expressed as –ve: negative result, +: weak intensity, ++: high intensity, and +++: very high intensity.

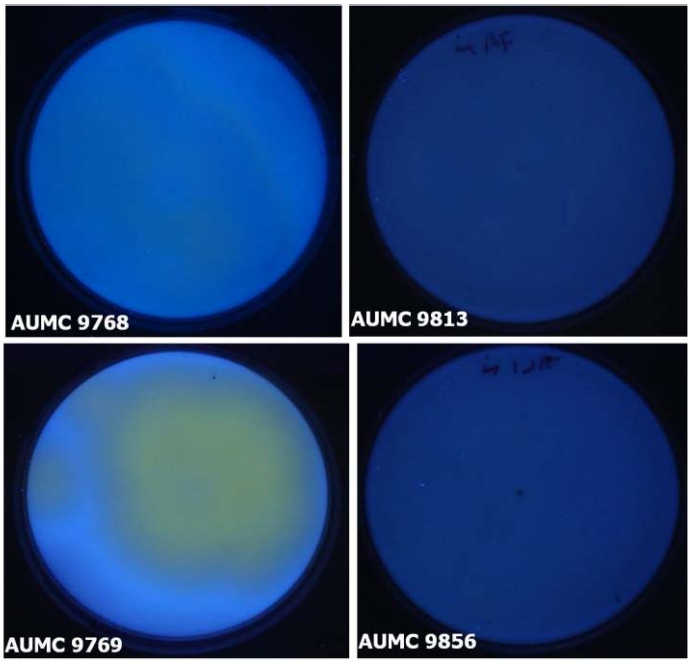

Figure 2 Blue fluorescence visible at 365 nm on coconut agar medium (CAM) for *Aspergillus flavus* strains Nos. AUMC 9768, AUMC 9813 & AUMC 9856 and greenish yellow fluorescence for AUMC 9769.

CONCLUSION

The number of fungal species recorded on wheat grains were higher than those recorded on corn and peanut. Only three genera (*Aspergillus*, *Penicillium* and *Fusarium*) were isolated from the three substrates with more propagules of *Aspergillus* being heavily contaminating peanut samples and more *Fusarium* and *Penicillium* propagules heavily contaminating corn samples. The aflatoxigenic species (*A. flavus*) was common on the three substrates on both isolation media. The results of flourescence at 365 nm of the 43 *A. flavus* and other 4 fungal strains recovered from peanut seeds, corn and wheat grains and grown on CAM agar plates, revealed that all *A. flavus* strains showed blue color with different intensities indicating aflatoxin B production, except one strain (AUMC 9796 originating from peanut seed) gave greenish yellow colour indicating aflatoxin G production, while the other 4 non-*A. flavus* strains showed negative results. Because of their deleterious effects, the incidence of moulds and levels of mycotoxins in foods should be frequently and routinely determined.

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VIABILITY OF *LACTOBACILLUS REUTERI* NCIMB 30242 DURING STORAGE IN FRUIT JUICE AND SOY BEVERAGE

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ABSTRACT

This study aimed to follow the viability of a probiotic culture in a soy beverage and in a fruit juice blend using quantitative PCR with propidium monoazide (PMA-qPCR). Free and microencapsulated (alginate and poly-L-lysine system) cells of *Lactobacillus reuteri* NCIMB 30242 were added at 10^8 CFU/mL in each food matrix and stored for 8 weeks at 4 or 8°C. In both matrices, viability losses during the 8 week storage period were less than 1 log CFU/mL. The pH of the fruit juices did not change during storage, but acidification occurred in the soy beverage, particularly when storage was carried out at 8°C. As a result, at a pH below 6.3, coagulation of the soy beverage occurred. It was found that qPCR could ascertain the total dead and viable population of *L. reuteri* in both food matrices. At day 1, the PMA-qPCR data in fruit juice were approximately 0.5 log cells/mL lower than in soy, which points to an effect of matrix itself on the qPCR analysis; the methodology was nevertheless successful in following the changes in *L. reuteri* viability during storage. Microencapsulation did not enhance the stability of the cultures.

Keywords: *Lactobacillus reuteri*, microencapsulation, food matrix, storage temperature PMA-qPCR

INTRODUCTION

Viability is still considered a critical component of the functionality of probiotic bacteria when they are consumed. Enumeration in plate counts (CFU) is the traditional method of evaluating viability (**Champagne *et al.*, 2011**), but newer methodologies are being developed. As a function of the nature of fluorochromes that enter (or not) into viable cells, flow cytometry can be used to rapidly assess bacterial viability (**Bunthof and Abee, 2002; Doherty *et al.*, 2010**). More recently, the propidium monoazide (PMA) reagent used in flow cytometry was applied to quantitative polymerase chain reaction (qPCR) to enumerate viable cells of probiotic bacteria in freeze-dried supplements (**Kramer *et al.*, 2009**), and in cheddar cheese (**Desfossés-Foucault *et al.*, 2012**). One of the goals of this study was to ascertain if the PMA-qPCR technique could be used in soy or fruit-based matrices as well.

Foods carrying probiotics are ideally marketed at 4°C, and it has been shown that incubation at room temperature will reduce stability during storage (**Klu *et al.*, 2012; Rozada *et al.*, 2009**). However, few teams have examined the effects of storage at 8 to 10°C which is sometimes termed "temperature abuse". Indeed, maintaining the cold chain between 2 and 4°C is not always respected in commercial environments, and increases may occur during shipping, display at the grocery store or in consumers' refrigerators. A few studies have shown that increasing the temperature just a few degrees into the 8 to 10°C range can significantly affect stability of probiotic bacteria during storage (**Mortazavian *et al.*, 2007; Rodgers and Odongo, 2002**). There is interest in further documenting the evolution of viability of probiotics in the "temperature abuse" range.

In the past, the development of functional foods with probiotics was mostly based on the selection of strains which could remain viable during the processing steps as well as during storage. In the future, however, in order to obtain health claims on the labels, strain selection will primarily be made on the basis of cultures which have demonstrated health benefits supported by clinical trials. As a result, companies will need to adapt processes, or food matrix characteristics, in order to enable sufficient viability of the selected strain. There are few reports, however, on strategies that can be used to select appropriate beverages for probiotics. *Lactobacillus reuteri* NCIMB 30242, a strain having demonstrated clinical benefits towards serum cholesterol levels and cardiovascular risk profile (**Jones *et al.*, 2012a and 2012b**), vitamin D status (**Jones *et al.*, 2013a**) and gastrointestinal health (**Jones *et al.*, 2013b**), is currently marketed as a supplement (LRC™, UAS Labs). This study was undertaken to ascertain if soy- or fruit-based matrices can be used for this purpose. Soymilk or soy beverages have often been suggested as vehicles for the delivery of probiotic bacteria to consumers. However, in most studies with soy-based matrices, the product is fermented. There are no data on the stability of *L. reuteri* in non-fermented soy beverages.

The aims of this study were therefore to select proper fruit and soy-based beverages to carry *Lactobacillus reuteri* NCIMB 30242, to examine the effect of storing at 4 and 8°C on viability of *L. reuteri* in the two food matrices and to compare traditional and qPCR technologies to follow its viability during storage at 4 or 8°C.

MATERIAL AND METHODS

Preparation of Free and Microencapsulated Lactobacilli

Lactobacillus reuteri NCIMB 30242 (LRC™, available at UAS Labs,Madison, WI, USA) was selected for this study because of its documented effects on serum cholesterol (**Jones *et al.*, 2012a and 2012b**) as well as its safety (**Branton *et al.*, 2010; Jones *et al.*, 2012c; Jones *et al.* 2012d**); these parameters are required to obtain a health claim status (**Health Canada, 2009**). *L. reuteri* NCIMB 30242, proprietary to Micropharma, was propagated in modified MRS broth in anaerobic conditions (95% nitrogen, 5% carbon dioxide) for 16 hours at 37°C. The cultures were then centrifuged at 3,300 *g* for 20 minutes at 4°C and the cell pellet was isolated by gently decanting the supernatant. Free *L. reuteri* NCIMB 30242 was prepared by re-suspending the cell pellet in a maltodextrin and cysteine solution under proprietary conditions (Micropharma Inc., Montreal, QC, Canada) and adding the suspension dropwise to liquid nitrogen for flash freezing. Microencapsulation was carried out as described in various patents (Prakash and Jones 2010; **Martoni *et al.* (2011**). Briefly, a *L. reuteri* NCIMB 30242 cell pellet was blended with a low-viscosity sodium alginate solution (Sigma, Alginic acid sodium salt from brown algae; Product Number A1112; viscosity: 4-12 cP, 1% in H_20 at 25°C) and encapsulation was performed using an Inotech Encapsulator IE-50 in a sterile environment. Microbeads were allowed to solidify in a 0.1 M calcium chloride solution followed by coating in sequential solutions of ε-poly-L-

lysine and sodium alginate with intermediate wash steps. The alginate-ε-poly-L-lysine-alginate (APA) microcapsules containing *L. reuteri* NCIMB 30242 were re-suspended in a solution of maltodextrin and cysteine and added dropwise to liquid nitrogen for flash freezing. The resulting frozen droplets of free and microencapsulated *L. reuteri* NCIMB 30242 were stored at -80°C until used.

Commercial Fruit Juices and Soy Beverages

The "Maxi mango" fruit blend was from Naked Juice (Glendora, Inc., Azusa, CA USA). The label stated that it was composed of mango, apple, orange, banana and lemon juices. The soy beverage was from Silk Original Vanilla fortified (WhiteWave Foods Company, Broomfield CO, USA). Three different production lots of each product were purchased.

Chemical Analyses of Juices and Soy Beverages

The pH of the products was assessed using an Accumet XL15 pH meter (Fisher Scientific, Montreal, QC, Canada). Redox level was evaluated using a portable pH meter (Oakton, Vernon Hills, IL, USA) equipped with a Combined PT-ring electrode (Metrohm, Herisau, Switzerland).

Inoculation of Beverages and Bacterial Enumeration by Plate Count*s*

When ready for use, a required amount of frozen culture was placed into a sterile test tube and incubated in a 37°C water bath for rapid thawing. This approach was used since rapid thawing is considered preferable to extended slow thawing at 4°C (**Champagne *et al.*, 2011**). The thawed cultures were then immediately used. In some instances where the frozen cultures had high bacterial densities, they were diluted in sterile commercial freezing medium (supplied by Microparma) prior to inoculation.

The thawed cell suspension (64 mL) of either free or microencapsulated (ME) bacteria was added to 1.6 L of product, in order to achieve 1 x 10^8 CFU/mL. For each culture, the products were fractioned into 16 x 100 mL portions and placed in 120 mL polyethylene bottles (Salbro bottle Inc., Woodbridge, ON, Canada). The headspace was flushed with N_2 and capped. Half of the bottles were placed at 4 °C while the remainder were placed at 8°C. Bottles were always kept capped and were only opened at the sampling time. Only one bottle served for microbial and pH analyses. The inoculation was repeated with three different lots.

The CFU analysis of ME cultures requires special procedures (**Champagne *et al.*, 2011**). In order to have a constant sample preparation method for free or ME cultures, a procedure designed to release cells from ME culture was used on both series of samples. These samples were collected at day 1 as well as at weeks 2, 4 and 8. They were analyzed for pH and viable cell counts using standard plate count assay and qPCR analyses. For CFU and qPCR, 10 mL of liquid containing free or ME *L. reuteri* NCIMB 30242 was added to 90 mL of citrate-peptone buffer (25.8 g/L trisodium citrate with 1 g/L peptone at pH 6.8) in a sterile stomacher bag and homogenized with the Stomacher® 400 Circulator (Seward, Worthing, West Sussex, UK) for 1 minute at 230 rpm and another minute at low speed (200 rpm). To allow dissolution of APA capsules, a hold period of ten minutes at room temperature was performed before proceeding to the second blending at low speed.

For CFU analyses, 1 mL of homogenized suspension was serially diluted in 9 mL of sterile buffer (8.5 g/L NaCl with 1 g/L peptone). Plate counts, as CFU, were performed in duplicates by pour plating the appropriate dilutions into MRS agar which was prepared by adding 15 g/L agar (BD-Difco) to MRS broth (Fluka; *Sigma*-Aldrich, St Louis, MO, *USA*) , sterilizing at 121°C for 15 min, cooling to room temperature and adding 1 % (v/v) of a filter-sterilized solution of 5 g/L L-cysteine HCL. The plates were incubated for 48 h at 37°C and under anaerobic conditions.

During bacterial enumerations based on qPCR, after the second blending, samples of 1 mL were taken in the stomacher bag for the propidium monoazide (PMA) treatment and DNA extraction and another sample for the comparison without PMA. The methodology follows below.

Optimization of PMA Quantitative PCR

Propidium Monoazide Treatment and DNA Extraction

The PMA treatment was carried out following the protocol of **Desfossés-Foucault *et al.* (2012)**. In the qPCR analysis, the treated and non-treated samples were processed with the same protocol except for the addition of PMA for the treated samples. Briefly, for the DNA extraction, cell pellets were suspended in 400 µL of buffer for enzymatic lysis (20 mM Tris HCl at pH 8, 2 mM EDTA, 12 g/L Triton X-100, 20 g/L lysozyme) and incubated at 37°C for 1 h. Then the QiaAmp DNA Stool Mini Kit (Qiagen, Mississauga, ON, Canada) was used with preliminary bead-beating step as proposed by **Desfossés-Foucault *et al.* (2012)**. To ensure the quality of DNA extraction, we performed two independent extractions for each sample. With DNeasy Blood and Tissue kit, we observed PCR inhibitors in soy beverage (results not showed). In using the QIAamp DNA Stool Mini Kit, containing an InhibitEX™ tablet that removes PCR inhibitors, as well as incorporating the kit recommendation to add bovine serum albumin (BSA) to the PCR mixture, we eliminated the problem of PCR inhibitors.

Primer Design and Verification of Primer Specificity

Proprietary strain specific primers targeting *L. reuteri* NCIMB 30242 were designed by Micropharma Limited and confirmed for specificity against 55 strains of *Lactobacillus* (*L. reuteri*, *L. fermentum*, *L. casei*, *L. acidophilus*, *L. delbrueckii* and *L. buchneri)* as well as strains of the genus *Bifidobacterium*, *Clostridium* and *Enterrococcus*. The primers were further tested against animal fecal samples demonstrating their specificity for *L. reuteri* NCIMB 30242. qPCR was performed in triplicate using an ABI PRISM 7500 Fast real-time PCR system with software version 2.0.5 (Appl. Biosystems, Foster City, CA, USA). Amplification and detection were carried out in 96-well plates with SYBR-Green PCR 2 X Master Mix (Appl. Biosystems). Two qPCR and PMA-qPCR series of analyses were carried out because two independent DNA extractions were performed for each sampling time. Each reaction was run in a final volume of 10 µL with 0.5 µM final concentration of each primer, 0.2 µg/µL of BSA and 2 µL of DNA sample. The amplification program consisted of 1 cycle of 95°C for 20 s, followed by 40 cycles of 95°C for 3 s, 55°C for 30 s, followed by melting curve. Negative and positive controls were included in each run.

Determination of PMA Treatment Efficiency, Detection Limit and Standard Curves

The efficiency of PMA treatment was verified by comparing qPCR results of different ratios of live and heat-killed cells added to the beverages, as proposed by **Taskin *et al.* (2011)** (Figure 1). For this analysis, 1000 µL of diluted sterilized beverages (1:10 in citrate/peptone buffer; 20 min in autoclave) were added to 1000 µL of mixture before treating with or without PMA. DNA extraction and qPCR amplifications were performed in following the same protocol as the other samples, and the cycle threshold of each sample was then compared to a standard curve made with PMA-treated live cells (10^9 to 10^3 CFU/mL). The detection limits were determined using the standard curve and treatment efficiency results.

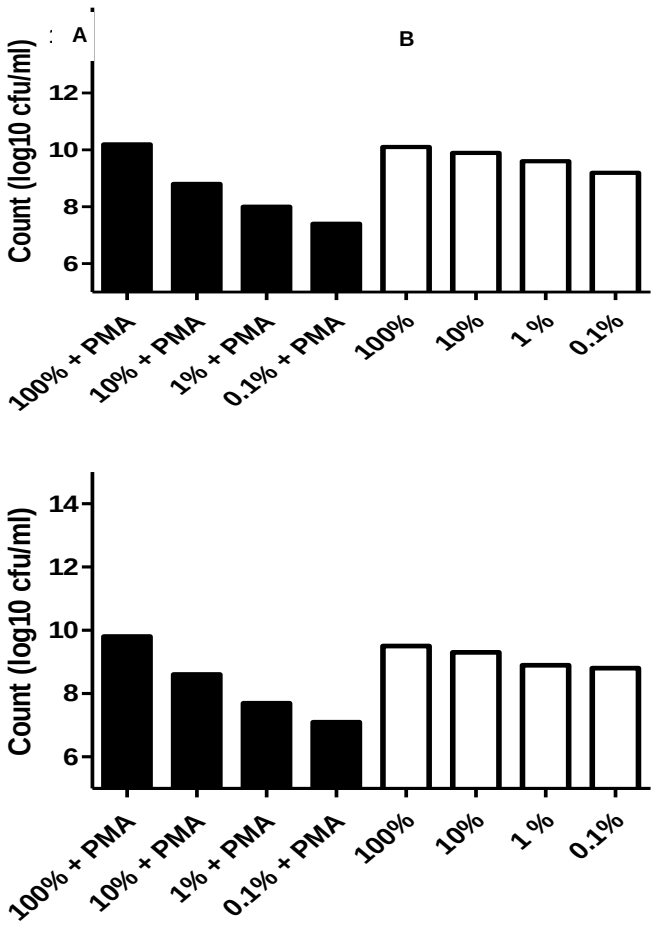

Figure 1 Effect of PMA treatment on PCR quantification of defined ratios of viable and heat-killed cells A) soy beverage and B) fruit juice.

Statistical Analyses

The inoculation of 100 mL beverages was made in three independent experiments, with three separate product lots. Statistical analyses on bacterial

counts during storage were performed with the JMP 7.0 software (SAS institute) using the "Proc Mixed" procedure. First, we carried out a full factorial test and then we removed the interactions where P was superior to 0.40. This approach allows reducing the model and thereby increasing the power of the test. A matched paired student test was also performed to verify the correlation between PMA-qPCR and viable count results. Correlations between different sets of data on the composition of the juice blends and their effect on bacterial growth, as well as between CFU and PMA-qPCR data, were carried out using the Spearman test from SigmaPlot version 12.5 software (Systat Software, Inc., San Jose, CA, USA).

RESULTS

Characteristics of the Food Matrices

The fruit juice blend had the following characteristics: pH 3.9, redox level of +123 mV. The soy beverage had a pH of 8.4 and a redox level of +109 mV. Therefore, for these two parameters, the main difference between the two products was pH.

Effect of Inoculation and 1 Day of Storage on CFU Counts

The viable counts one day after inoculation were on average 0.14 log (38%) CFU/mL higher in the soy beverage than in the fruit juices. This small difference was found to be statistically significant (P = 0.008), which is rather unusual with viable counts (Table 1). Evidently, the inoculation method, which consisted of direct inoculation with a frozen concentrate, enabled a good standardization of the experimental procedures. The higher CFU in the soy beverage was linked to a slight viability loss in the fruit juice.

Evolution of pH During Storage

The pH values of the soy beverage went down during storage in every condition (Figure 2). Statistical analyses showed significant effects of encapsulation (treatment), storage temperature and time (Table 1). The effect of time is quite logical, and it was to be expected that acidification would occur gradually.
At what is considered an abuse storage temperature, there was a significantly higher drop in pH during storage at 8°C. As a result of all these interactions, the pH of ME 8°C in the soy beverage decreased rapidly to reach a value around pH 6. The decrease profile of ME 4°C and free 8°C is quite similar (not statistically different).
A very different picture emerged in the case of the fruit juice. For this matrix, the pH remained almost the same over 2 months storage time (Figure 3). Overall, the pH was not significantly affected by storage temperature or microencapsulation (Table 1).

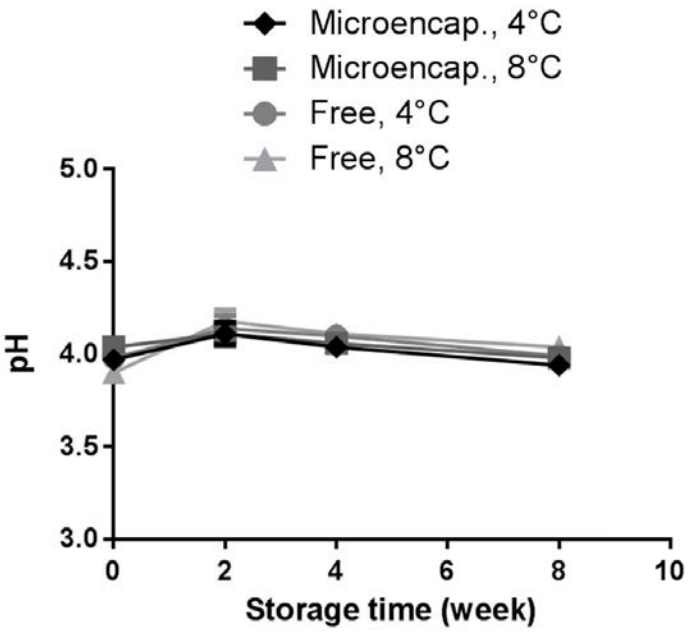

Figure 2 Evolution of pH in soy beverage containing *Lactobacillus reuteri* NCIMB 30242 during storage.

Effect of Storage for Eight Weeks on Plate Counts (CFU)

In soy beverage, the loss in viability over 8 weeks was limited to 0.2 log $CFU.mL^{-1}$ with free cells (Figure 4A). Although small in size, this drop in CFUs during storage became statistically significant after 4 weeks of storage. No major effect of incubating at 8°C on CFU counts was noticed. However the viability loss of the ME culture was about twice the level observed for free cells.
The CFU loss during storage in the fruit juice beverage was similarly acceptable (Figure 5A), since they were not greater than 1 log CFU per mL. There were nevertheless statistically significant effects of time, incubation temperature and culture format on CFU readings. Incubation at the "abuse" temperature of 8°C increased the rate of viability losses, but this was limited to about 0.1 log CFU/mL. The effect of the state of the culture was of greater significance. Viability losses of free cells over the 8 week storage period were limited to about 0.3 Log CFU/mL while the ME culture showed a viability drop of up to 1 log CFU/mL (Figure 5A).

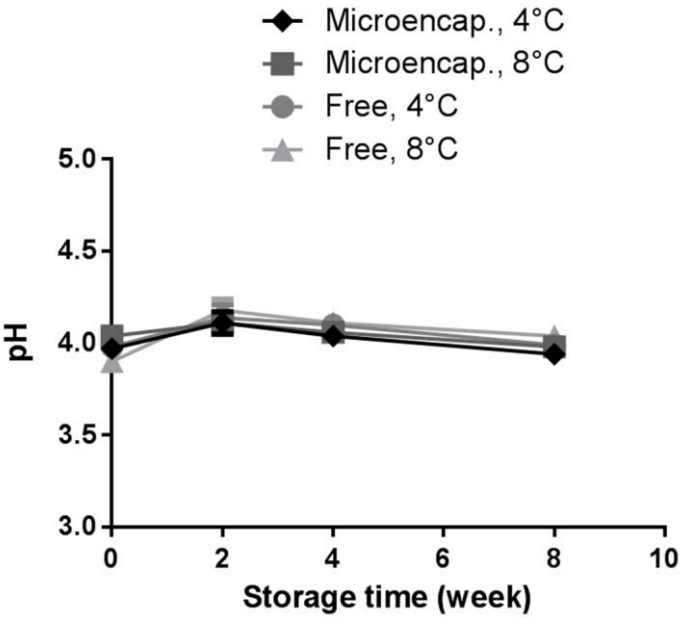

Figure 3 Evolution of pH in fruit juice containing *Lactobacillus reuteri* NCIMB 30242 during storage.

Effect of Storage on Viable + Not Cultivable Counts (PMA-qPCR)

In soy beverage, there was a significant effect (P = 0.005) of the cell state on PMA-qPCR viability, while the effect of storage time showed only a tendency to statistical significance (P = 0.08) and the overall effect of storage temperature was negligible (P = 0.61). In this series of assays in the soy beverage, the ME culture stored at 8°C proved to be the least stable (Figure 4B).
A different situation was noted with the fruit juice blend. There were no effects of cell state (P = 0.83) or storage time (P = 0.09), but incubation temperature affected PMA-qPCR viability levels (P = 0.03). As was observed in plate counts (CFUs), a slight reduction in PMA-qPCR viability levels occurred with ME culture in fruit juice stored at 8°C (Figures 4 and 5).

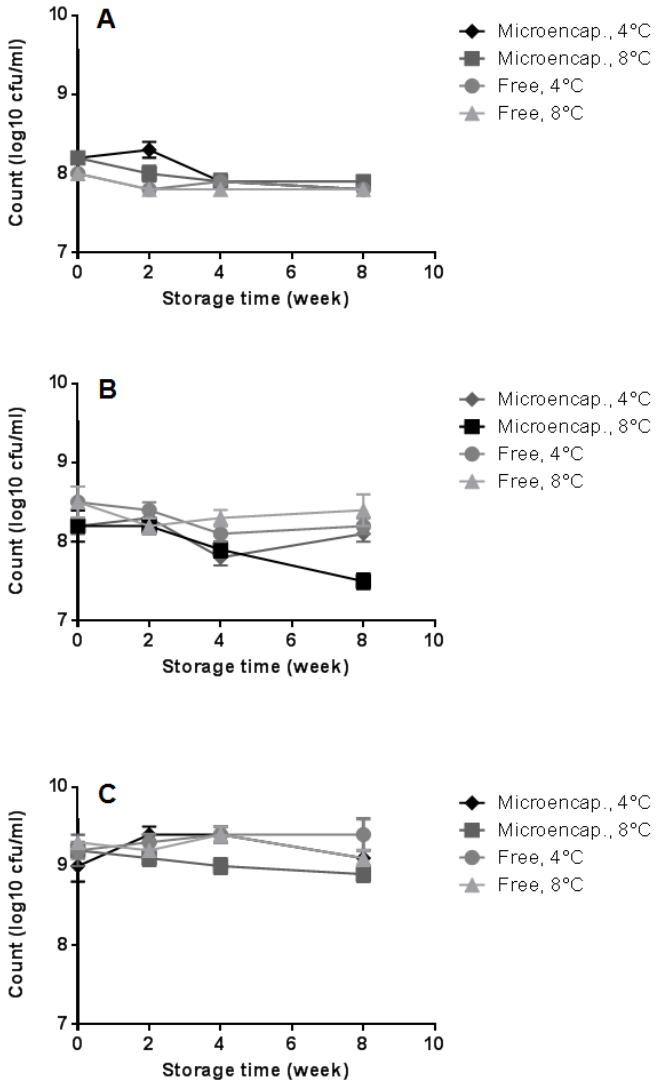

Figure 4 Number of *Lactobacillus reuteri* NCIMB 30242 in soy beverage during storage quantified by different methods A) plate count, B) PMA-qPCR and C) qPCR.

Overall, the CFU and PMA-qPCR data were in agreement, since the correlation coefficient (R = + 0.55) was statistically significant (P = 0.001). Therefore the PMA-qPCR could successfully be used to evaluate viability variations in the food matrices.

Effect of Storage on Viable + Dead Counts (qPCR)

With the qPCR method, without addition of PMA, we obtained, at day 1, higher values of approximately 1 log (0.9 log for soy and 1.3 log for fruit) (Figures 4B, 4C, 5B, 5C) than for PMA-qPCR. This is explained by the fact that, without the PMA, the extracted DNA also contains that of dead cells. The aspects on viability loss resulting from inoculation in the matrix itself were discussed previously, and could account for up to 0.2 log of the differences between qPCR and PMA-qPCR values, suggesting that the cell suspension used to inoculate the food matrices contained a significant proportion of non-viable cells.
When comparing ME and free-cell values at T = 1 day, data in both qPCR and PMA-qPCR series of data (paired t test) no effect of ME (P = 0.44) was noted. However, the food matrix had a significant effect on the qPCR data. Indeed, theoretically, qPCR values in fruit juice and soy should be identical. In practice, at T = 1 day, the data in fruit juice were approximately 0.5 log cells/mL lower than in soy (Figures 5B and 5C).

Visual Appearance of the Soy Beverages

While the fruit juice blend was stable in pH and texture over 8 weeks in all conditions, in the soy beverage there were instances where coagulation of the gel appeared. It was not established at what exact pH coagulation could be visually noticeable, but all soy beverage samples that had a pH above 7.16 were in the liquid state, while precipitation was noted in those which were at pH 6.3 or below. For samples with free cells stored at 4°C, a storage period of 8 weeks was possible. However, the three other treatments showed some coagulation after 4 weeks and could potentially benefit from a lower initial inoculation of *L. reuteri*. No sensory tests on flavour were carried out, but these data on texture suggest that further tests are warranted on this important sensory aspect.

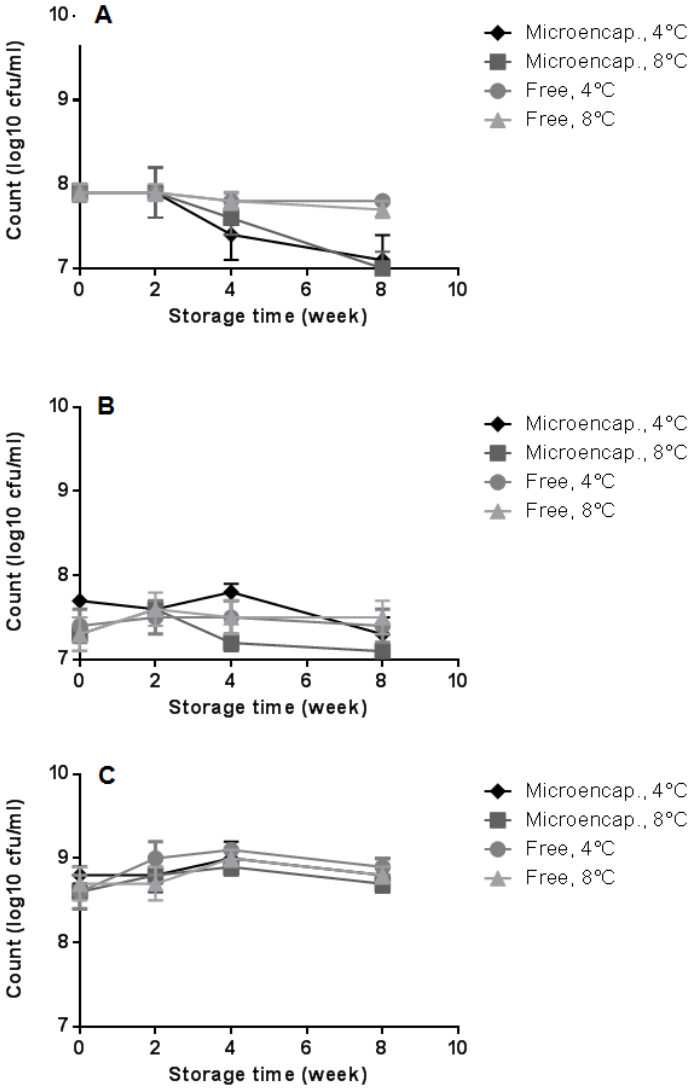

Figure 5 Number of *Lactobacillus reuteri* NCIMB 30242 in fruit juice during storage quantified by different methods A) plate count, B) PMA-qPCR and C) qPCR.

Table 1 Analysis of variance of the effects of experimental conditions on the evolution of pH in fruit juice and soy beverage. Data are the probability (P) of an absence of effect on pH during storage.

Source	Soy beverage	Fruit juice
Microencapsulation (ME)	< 0.0001	0.3375
Temperature	< 0.0001	0.4843
Time	< 0.0001	0.0005
ME * Temperature	0.0002	
ME * Time	< 0.0001	
Temperature * Time	< 0.0001	
ME * Temperature * Time	< 0.0001	

DISCUSSION

Most scientific studies designed to evaluate the effect of changes in the composition of a food matrix on its subsequent spoilage are carried out by modifying “one parameter at a time” (such as pH) or by changing “one ingredient at a time” (for example salt). Unfortunately, it becomes difficult to compare data from two very different matrices in two separate studies, because probiotic strains, experimental equipment of analytical methods differ between the two publications. This study was deliberately designed to compare two matrices having many chemical differences. Such an approach enabled the examination of a common set of experimental parameters (packaging conditions, storage temperature, analytical methods, probiotic strain, ME) on the viability of a probiotic culture as well as how it can spoil the food matrix.

Effect of pH on Viability

On the basis of CFU values immediately following inoculation or after day 1, data suggest that inoculation in fruit juice would need to be between 0.14 and 0.5 log CFU/mL higher than in the soy product to obtain similar viable counts at the end of storage. A rapid viability loss upon inoculation in juice has been reported (**Reid *et al.*, 2007**), but this was not the only reason for the differences between soy and fruit juice CFU data at day 1. Indeed, CFUs in the soy beverage after day 1 are slightly higher that would theoretically be found after inoculation. This suggests slight growth.

The stability of probiotics during storage in food matrices is strongly influenced by pH, (**Kailasapathy *et al.*, 2008; Nualkaekul and Charalampopoulos, 2011**) and the redox level (**Bolduc *et al.*, 2008**). Even when blends of various juices enable the product to be adjusted at pH 4.2, viability losses of up to 5 log CFU/mL occur over 60 days of storage, as a function of strain and species (**Saarela *et al.*, 2006; Champagne and Gardner, 2008**). In this study, *L. reuteri* showed good stability in a juice blend during 60 days of storage, which was in line with the data on another strain of the same species (**Champagne and Gardner, 2008**). These results suggest that *L. reuteri* is a good candidate for enrichment of fruit juices.

Studies on the effect of storage pH on viability have examined acid environments. To our knowledge, no stability studies have been made for *L. reuteri* at alkaline pH levels, and the high pH of 8.6 of the soy beverage was a concern. The *L. reuteri* NCIMB 30242 culture was more stable in the soy beverage than the fruit juice. Evidently, a slightly alkaline environment is less detrimental that an acid one for stability of this strain during storage.

Effect of Probiotics on Matrix pH

No data exist on the effect of high inoculation levels of *L. reuteri* (10^8 CFU/mL) in unfermented soy beverages. In cow milk however, which is arguably the closest comparative food matrix, considerable variations in pH can occur during storage as a function of strain, inoculation level and storage temperature (**Saarela *et al.*, 2006; Bolduc *et al.*, 2006; Sanders *et al.*, 1996**). In a study involving eight strains of bifidobacteria inoculated at approximately 7.2 log CFU/mL, the pH of milk after a two week incubation at 4°C varied between 6.68 (e.g. no change) and 5.31 as a function of strain and redox level of milk (**Bolduc *et al.*, 2006**). **Sanders *et al.* (1996)** also reported acidification to pH 5.5 over 3 weeks storage, while **Saarela *et al.* (2006)** did not find any. In light of this literature, it appears that the *L. reuteri* NCIMB 30242 culture used in this study is not a highly-acidifying culture under refrigeration conditions if kept at 4°C. Since data show that there is nevertheless acidification, industry might take advantage of the opportunities allowing for adjustment in buffering capacity of the products. Indeed, a soy beverage would be more sensitive to a pH drop than milk because its buffering capacity is lower (**Zare *et al.*, 2011**).

Microencapsulation

Acidification tended to be higher with the ME culture, which is in line with the observations of **Truelstrup-Hansen *et al.*, (2002)** in unfermented milk. The greater acidification of the soy beverage with the ME cultures, as compared to free cells was, nevertheless, unexpected. With the exception of dry sausage fermentation (**Kearney *et al.*, 1990**), at optimum fermentation temperatures (about 37°C for probiotics), ME cultures in alginate gels have lower specific acidifying properties than free cells (**Champagne *et al.*, 1988**). The potential differences in physiological state between free and ME cells was examined by comparing, in paired t tests, the CFU and PMA-qPCR data of samples. There was no significant difference, which suggests that, the free and ME cells appeared similar with respect to membrane properties and culturability. Therefore, the differences between free and ME in this study should be investigated further.

Since CFUs in soy beverage were higher at day 1 than those at inoculation, it must be assumed that some growth occurred. Therefore, the higher values in CFU with free cells, in comparison to ME cultures, might partially reflect better growth of the free cells following inoculation rather than a lack of protective effect of ME.

Effect of storage temperature

Although the products should be kept between 1 and 4°C, their temperature often rises during shipping, or during storage in retail outlet refrigerators (**Juneja *et al.*, 2006**). Even between 0 and 10°C, there can be an increase in acidification during storage (**Micanel *et al.*, 1997)** and lesser stability of probiotics **(Mortazavian *et al.*, 2007).** Since pH affects the viability of probiotics during storage (**Roy, 2005; Kailasapathy *et al.*, 2008**), as well as sensory properties, evaluation of the evolution of pH during this period is warranted.

In soy, storing at 8°C promoted acidification. In contrast, the pH of fruit juice inoculated with free or ME cells remained stable during storage at both temperatures. This is in agreement with data in the literature in instances where pH of the various juices were in the 3.7-4.1 range (**Elizaquível *et al.*, 2011; Saarela *et al.*, 2006**). Presumably, the highly sub-optimal pH environment strongly reduced metabolic activities and prevented an abuse storage temperature to become a problem. This study therefore shows that the nature of the food matrix influences the impact of inadequate storage temperature on the spoilage which results from the presence of probiotic cultures.

PMA-qPCR for bacterial counts

The CFU methodology is a classical technique, but it requires an extensive incubation period. New techniques which provide more rapid responses are on viable and total counts are required. This study was very appropriate to validate the recently-developed PMA-qPCR methodology. Indeed, the state of the cells could be affected by the food matrix (pH, fat, protein etc.) as well as by microencapsulation, ant it provided unique comparative conditions to assess the PMA-qPCR technique. This study confirmed the data of **Desfossés-Foucault *et al.* (2012)** to the effect that PMA-qPCR is a reliable methodology is assessing viable counts in a food matrix. It is the first study, however, to examine the effect of very different food matrices on its results. In food microbiology, it is well known that the nature of the food matrix will influence the results of microbial analyses. For example, the CFU analytical procedure carried out on yogurt would not be appropriate with cheese. In yogurt, sample homogenization can be carried out at room temperature with a peptone buffer as diluent. Such an approach would result in an underestimation of CFUs from a cheese sample, since homogenization at 40°C in a citrate buffer is best with cheese (**Duncan *et al.*, 2004**) because it enables a more extensive release of cells from curds.

The good correlation between CFU values and PMA-qPCR in both matrices, means that PMA-qPCR can follow the viability of *L. reuteri* NCIMB 30242 in fruit juice and a soy beverage. However, for identical inoculation levels, qPCR data in soy were slightly higher than those in fruit juice. This could be due to an effect of the matrix itself on the qPCR analysis, or greater autolysis of cells in the fruit juice. It could also be hypothesized that some growth occurred in the soy beverage during day 1. Further studies on the nature of the effect of the food matrix on qPCR data are therefore warranted.

***Lactobacillus reureti* NCIMB 30242 for Foods**

Data from this study were compared with those in the literature in order to ascertain the position of *L. reuteri* NCIMB 30242 as a strain of commercial interest. Again, since there are no data in non-fermented soy beverages with *L. reuteri*, our comparative examination was carried out on data from unfermented milk. In such products, loss of viability of other probiotics is typically less than 1 log CFU/mL (**Sanders *et al.*, 1996; Saarela *et al.*, 2006; Truelstrup-Hansen *et al.*, 2002**). However, as a function of strains and how they are prepared, viability losses varying from none (**Saarela *et al.*, 2006; Truelstrup-Hansen *et al.*, 2002**) to 5 log CFU/mL (**Bolduc *et al.*, 2006; Truelstrup-Hansen *et al.*, 2002**) of bifidobacteria have been reported. In light of these literature data, *L. reuteri* NCIMB 30242 can be considered as a stable culture in a soy beverage and compares favourably to those observed in the literature under similar conditions.

CONCLUSION

Soy beverages and fruit juices can successfully be used to deliver viable *L. reuteri* probiotics since losses were lower than 1 log CFU per mL. In addition, if stored in appropriate refrigerated conditions with an appropriate culture format, *L. reuteri* NCIMB 30242 can be delivered at a more than 10 billion cells per 250 mL portion without negatively impacting the texture of foods. This is 10 times more than most food products on the market, which currently deliver 1 billion cells per portion. Most studies on the stability of probiotics during storage in foods examine the effect of strain or ingredients in a single matrix. This study showed that developing a new food product with a given strain holds many challenges.

This study also contributes novel observations for the sector: 1) qPCR and PMA-qPCR can be used to ascertain the total dead and viable population of *L. reuteri* NCIMB 30242 in fruit juices and soy beverages, and data of the latter are in agreement with CFU, 2) the food matrix affects viability readings of *L. reuteri* NCIMB 30242, as verified by PMA-qPCR, 3) microencapsulation was not effective in enhancing viability during storage in the fruit juice ($P = 0.3375$), 4) increasing the temperature from 4°C to 8°C only has minor effects on growth or viability losses over 8 weeks of storage but 5) when the soy beverages are inoculated at 10^8 CFU/mL, significant drops in pH occur during storage and particularly at 8°C ($P < 0.0001$).

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SOUND STIMULATION CAN INFLUENCE MICROBIAL GROWTH AND PRODUCTION OF CERTAIN KEY METABOLITES

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ABSTRACT

Effect of two different audible sound (music) patterns on six different microorganisms was investigated. Both the sound patterns namely *Ahir Bhairav* (172-581 Hz) and *Piloo* (86-839 Hz) were able to significantly affect microbial growth and production of certain key metabolites by the test microbes. Faster uptake of glucose from the growth medium by *Brevibacillus parabrevis* and *Saccharomyces cerevisiae* was observed under the influence of sound. Production of quorum sensing-regulated pigments, prodigiosin and violacein, respectively by *Serratia marcescens* and *Chromobacterium violaceum* was also notably affected by sound treatment. Further investigation to decipher molecular basis of microbial response to sound is warranted to understand the mechanism how audible sound interacts with microbial cells, and how the microbial population(s) modulate their behavior in response to sound stimulation.

Keywords: Sound stimulation, Music, Growth, Pigment, Membrane, Quorum sensing

INTRODUCTION

Living cells and organisms can sense changes in various environmental factors in their surroundings, and respond accordingly. Effect of many of the environmental factors such as pH, temperature, osmotic pressure, light, etc. has been well characterized. However sound remains one such factor, which despite being widely present in the growth environment of almost all living systems, whose effect on living cells/organisms has not been characterized that well. Sound's effect on higher organisms having some sort of sound-sensing organ (e.g., ears in animals) is of obvious nature. There have been few reports **(Hassanien *et al.*, 2014)** describing the effect of noise and/or music on plants and animals. Not many such systematic investigations describing influence of sound on microorganisms are available. Much remains to be investigated on how microorganisms interact with external sound, and how they behave when placed in an external sound field. This is a relatively nascent area of scientific research. Certain earlier reports **(Matsuhashi *et al.*, 1998)** have indicated microorganism's ability to produce, sense, and respond to sound. However most reports are concerning the sound beyond human audible range i.e. 20-20,000 Hz. In the present work, we have focused on the effect of audible sound (composed of multiple frequencies) on selected microorganisms, when the sound is provided in form of a particular pattern i.e. music.

MATERIALS AND METHODS

Test organisms

List of the prokaryotic and eukaryotic microorganisms employed in this study, and the parameter(s) on which the influence of sound was investigated, is provided in Table 1. All the test strains were procured from Microbial Type Culture Collection (MTCC), Chandigarh. All media/media components were from HiMedia, Mumbai.

Table 1 Test organisms

No.	Organism	MTCC code	Growth Medium	Incubation temperature (ºC)	Incubation time (h)	Parameter(s) tested
1.	*Chromobacterium violaceum*	2656	Nutrient broth	35	48 (under static condition)	Growth and violacein production
2.	*Serratia marcescens*	97	Nutrient broth	28		Growth and prodigiosin production
3.	*Xanthomonas campestris*	2286	Tryptone yeast extract broth (supplemented with $CaCl_2$)	Room temperature	72 (with intermittent shaking)	Growth and exopolysaccharide (EPS) production
4.	*Brevibacillus parabrevis*	2708	Broth containing CMC-Na[#]	35	72 (with intermittent shaking)	Growth and cellulase activity
5.	*Lactobacillus Plantarum*	2621	MRS broth	Room temperature	48 (under static condition)	Growth and pH
6.	*Saccharomyces cerevisiae*	170	Glucose yeast extract broth	Room temperature	48 (static)	Growth and alcohol production

[#]CMC: carboxymethyl cellulose

Sound treatment:

Sound stimulation of the test cultures was executed as described in our previous study **(Sarvaiya and Kothari, 2015)**. Inoculum of test organism from its activated culture was prepared in sterile normal saline and was standardized to 0.5 McFarland turbidity standard. The tubes with growth medium after inoculation were put into a glass chamber (Merck; 225 ×225 ×125 cm). A speaker was placed in this glass chamber at a distance of 15 cm from the inoculated test tubes/flasks, from which the sound was delivered (sound delivery was provided throughout the period of incubation). This glass chamber was covered with a glass lid, and multiple layers of cloth and paper were used to cover the lid. This was done to prevent any possible leakage of sound from the chamber, and also to avoid any possible interference from external sound. Similar chamber was used to house the control (not stimulated with music) group test tubes. One speaker was also placed in the glass chamber used for the control test tubes at a distance of 15 cm, where no electricity was supplied and no sound was generated. Frequency of the test sound was analyzed by NCH WavePad Sound Editor Masters Edition v. 5.5. Of the two music types used, raag *Ahir Bhairav* (sound-I) was found to be composed of sound falling in the frequency range 150-7811 Hz, of which the range 172 - 581 Hz was dominant (Figure 1). Similarly, frequency range of the second test music raag *Piloo (Teentaal;* sound-II) was determined to be 43-5620 Hz, of which the range 86-839 Hz was dominant (Figure 2). Source for both these sound patterns was the commercially available musical disc titled *Call of the Valley* (Saregama India Ltd., Kolkata). Intensity of the sound, measured with a sound level meter (acd machine control Ltd.) at a distance of 15 cm from the speaker was 70-90 dB (in case of *Ahir Bhairav*), and 85-110 dB (in case of *Piloo*). Absence of detectable sound in the control chamber was ensured using decibel meter.

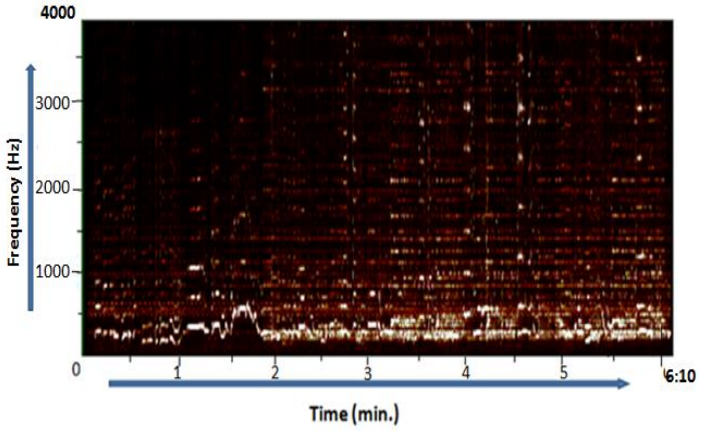

Figure 1 Frequency distribution over time for the *raag Ahir Bhairav*

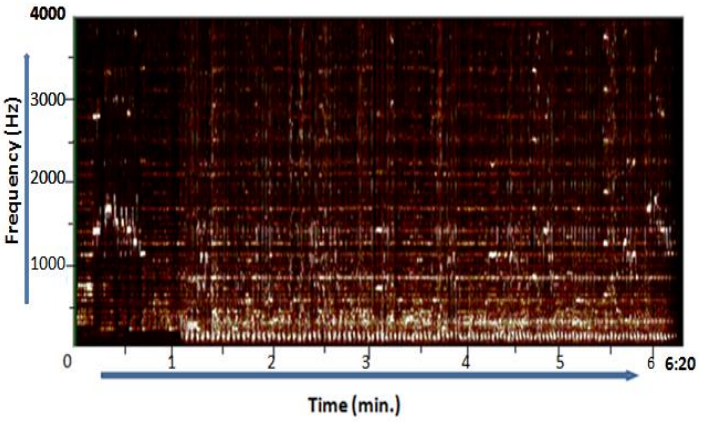

Figure 2 Frequency distribution over time for the *raag Piloo (Teentaal)*

Violacein extraction and estimation

After measuring growth by recording OD at 660 nm using spectrophotometer (Agilent- Carry 60), violacein extraction **(Choo *et al.*, 2006)** was done from *C. violaceum* culture. Briefly, 3 mL of the culture broth was centrifuged (REMI CPR-24 Plus) at 12,000 rpm (13,520 g) for 15 minute at 25^{0}C, and the resulting supernatant was discarded. The remaining cell pellet was resuspended into 3 mL of DMSO purchased from Merck (Mumbai), and incubated at room temperature for 30 min, followed by centrifugation at 12,000 rpm for 15 min. The violacein extracted in the supernatant was estimated by measuring OD at 585 nm.

Prodigiosin extraction and estimation

After quantifying growth at 660 nm, prodigiosin extraction was carried out from *S. marcescens* culture as described by Pradeep *et al.* **(2013)**. Briefly, 3 mL of the culture broth was centrifuged at 12,000 rpm for 15 min, and the resulting supernatant was discarded. The remaining cell pellet was resuspended in 3 mL of acidified methanol (4 mL of HCl into 96 mL of methanol; Merck), followed by incubation in dark at room temperature for 30 min. This was followed by centrifugation at 12,000 rpm for 15 min at 4°C. Prodigiosin in the resulting supernatant was estimated by measuring OD at 535 nm.

Alcohol estimation

Alcohol was estimated photometrically. *S. cerevisiae* culture broth was centrifuged at 12,000 rpm for 15 min, and the supernatant was used for alcohol estimation **(Williams & Darwin, 1950).** 1 mL of the supernatant was mixed with 15 mL of distilled water, and 25 mL of potassium dichromate reagent, followed by 10 mL of additional distilled water. Then incubation was carried out in water bath at 60°C for 30 min, followed by measurement of OD at 600 nm. Standard curve was prepared using absolute ethanol procured from Eureka, Ahmedabad at 2–10% v/v.

EPS quantification

Following estimation of growth by measuring OD at 660 nm, culture broth was subjected to centrifugation at 7,500 rpm (6,600 g) for 15 min, and the cell free supernatant (CFS) was used for EPS quantification using the method described in Li *et al.*, **(2012)** with some modification. Briefly, 40 mL of chilled acetone (Merck) was added to 20 mL of CFS, and allowed to stand for 30 min. The EPS precipitated thus was separated by filtration through pre-weighed Whatman # 1 filter paper (Whatman International Ltd., England). Filter paper was dried at 60°C for 24 h, and weight of EPS on paper was calculated.

Glucose uptake assay

Concentration of glucose present in the medium (after inoculation of test organism) was estimated at different time intervals, employing the photometric method using dinitrosalicylic acid (DNSA) **(Nigam and Ayyagari, 2008).** For this experiment *Brevibacillus parabrevis* was inoculated into a growth medium containing 0.5 g/L glucose, 0.5 g/L NaCl, 0.5 g/L beef extract, 0.5 g/L yeast extract, and 0.5 g/L peptone. Medium for *S. cerevisiae* contained 10 g/L glucose, 5 g/L yeast extract and 10 g/L peptone.

Estimation of cellulase activity

B. parabrevis was grown in a CMC supplemented broth (0.5 g/L peptone, 0.5 g/L NaCl, 0.5 g/L Beef extract, 0.5 g/L Yeast extract, 20 g/L CMC-Na). The cell free supernatant obtained from *B. parabrevis* culture after centrifugation of the culture broth (10,000 rpm; 9,390 g) was used as crude cellulase preparation. 0.5 mL of the supernatant was mixed with 0.5 mL of 1% carboxymethyl cellulose (CMC-Na salt; Merck), followed by incubation at 50°C for 30 min. The amount of glucose released as a result of cellulase activity was quantified using DNSA colorimetric assay. The international unit (IU) of the cellulase was calculated as: IU= [(µg of glucose)/ 180 (molecular weight of glucose) x 30 (incubation time) x 0.5 (aliquote)] **(Nigam and Ayyagari, 2008)**.

Statistical analysis

All the experiments were performed in triplicate, and measurements are reported as mean ± standard deviation (SD). Statistical significance of the data was evaluated by applying *t*-test using Microsoft Excel®. Data with *p* values less than 0.05 was considered to be statistically significant.

RESULTS AND DISCUSSION

Raag Ahir Bhairav

Results regarding effect of sound-I are shown in Table 2. Growth and the particular test parameter(s) in case of all the test organisms experienced an enhancement under the influence of sound-I, except *S. marcescens*. The latter synthesized considerably lower pigment, when incubated in presence of music. This organism was earlier also found to experience a reduction in growth and prodigiosin production under influence of sound (*raag Kirwani*) corresponding to 38-689 Hz **(Sarvaiya and Kothari, 2015)**. This range of frequency includes the one corresponding to sound-I used in this study, however relative proportion of different frequencies is likely to be different in different music patterns, and accordingly their effect and its magnitude may vary. During another study conducted by us (using sound corresponding to 41-645 Hz; data yet unpublished) *S. marcescens* was found to produce almost 1.5 times higher prodigiosin despite a small reduction in growth. Besides *S. marcescens*, two more gram-negative

bacteria (*X. campestris* and *C. violaceum*) were used in this study, and both were found to grow better under the influence of sound-I, with simultaneous enhancement in synthesis of their respective test metabolites. Both the pigment producing organisms used in this study were affected by sound stimulation, but their response was of opposite nature. Whereas *S. marcescens* responded to sound stimulation negatively, *C. violaceum* showed better growth and pigment (violacein) production in response to the same sound treatment. Production of both these pigments (prodigiosin and violacein) is known to be regulated by quorum-sensing in the producing bacteria **(Wei *et al.*, 2006; Zinger-Yosovich *et al.*, 2006; Morohoshi *et al.*, 2007)**. Magnitude of effect of sound stimulation on EPS production and prodigiosin synthesis respectively in *X. campestris* and *S. marcescens* was much higher than its effect on growth of these organisms, indicating that the cellular machinery related to growth was affected differently than that related to synthesis and/or secretion of EPS and prodigiosin.

Table 2 Effect of *raag Ahir bhairav* on test organisms

Organism	Growth (OD_{660})			Prodigiosin (OD_{535})			Prodigiosin unit (OD_{535}/OD_{660})		
	Control (Mean±SD)	Experimental (Mean±SD)	% change	Control (Mean±SD)	Experimental (Mean±SD)	% Change	Control	Experimental	%Change
S. marcescens	0.67±0.00	0.64±0.00	-4.32**	0.52±0.03	0.36±0.03	-30.76**	0.77	0.56	-27.27
	Growth (OD_{660})			**Violacein production (OD_{585})**			**Violacein unit(OD_{585}/OD_{660})**		
C. violaceum	Control (Mean±SD)	Experimental (Mean±SD)	% Change	Control (Mean±SD)	Experimental (Mean±SD)	% Change	Control	Experimental	% Change
	0.75±0.01	0.78±0.01	4.0*	0.37±0.01	0.41±0.004	10.81*	0.49	0.52	6.12
	Growth (OD_{660})			**EPS Production (g/L)**			**EPS Production per unit OD (g/L)**		
X. campestris	Control (Mean±SD)	Experimental (Mean±SD)	% Change	Control (Mean±SD)	Experimental (Mean±SD)	% Change	Control	Experimental	% Change
	0.45±0.01	0.48±0.01	6.66*	3.56±0.11	4.70±0.20	32.0**	7.91	9.79	23.76
	Growth (OD_{660}) (1:1 dilution)			**Alcohol production (% v/v)**			**Alcohol production per unit OD (% v/v)**		
S. cerevisiae	Control (Mean±SD)	Experimental (Mean±SD)	% Change	Control (Mean±SD)	Experimental (Mean±SD)	% Change	Control	Experimental	%Change
	1.23±0.01	1.34±0.01	8.94**	1.58±0.01	1.74±0.00	10.06*	1.29	1.30	0.77
	Growth (OD_{660})(1:1 dilution)			**pH**					
L. plantarum	Control (Mean±SD)	Experimental (Mean±SD)	% Change	Control (Mean±SD)	Experimental (Mean±SD)	% Change			
	1.14±0.00	1.21±0.00	6.14**	4.26±0.05	4.03±0.05	-5.39**			
	Growth (OD_{660})			**IU**					
B. parabrevis	Control (Mean±SD)	Experimental (Mean±SD)	% Change	Control (Mean±SD)	Experimental (Mean±SD)	% Change			
	0.48±0.00	0.57±0.02	18.75**	0.021±0.001	0.026±0.001	23.80**			

***p≤0.01, *p≤0.05, '-'*minus sign indicates a decrease over control

Both the gram-positive bacteria (*B. parabrevis* and *L. plantarum*) exhibited a higher growth in presence of music, with almost similar magnitude of change in their respective test parameters. A little higher (5.39%) reduction in the pH of the growth medium of *L. plantarum* under the influence of sound may be owing to somewhat enhanced lactic acid production by this bacterium when induced by sound stimulation. Better growth of *B. parabrevis* under the influence of sound stimulation may be attributed to its higher (23.80%) cellulase activity enabling it to utilize cellulose (the principal carbon source in the medium used) more effectively. *S. cerevisiae* also registered higher growth and alcohol production when subjected to sound stimulation. During our previous study **(Sarvaiya and Kothari, 2015)** we found membrane permeability of the test microorganisms to increase in response to sound stimulation. To investigate whether the altered growth of the test organisms exposed to sound treatment in this study is due to any change in movement of key molecules across the cell membrane, we measured the rate of glucose utilization by *S. cerevisiae* and *B. parabrevis* when incubated in presence of the test sound-I, by quantifying the extracellular glucose present in the growth medium at different time intervals. Sound stimulated culture of *B. parabrevis* was found to utilize glucose at a faster rate than control culture (Figure 3), and accordingly its growth was faster under the influence of sound. At the end of 24 h of incubation extracellular glucose concentration in the experimental tubes was 11.53% lesser than the control tubes and the corresponding increase in growth amounted to 15.94%. Similarly, *S. cerevisiae* could also utilize glucose faster under the influence of sound. At the end of 48 h of incubation the extracellular glucose concentration in the experimental tubes was 17.39% lesser than that in control tubes, and the corresponding increase in growth and alcohol production was found to be 26.44% and 24.21% respectively (Figure 4).

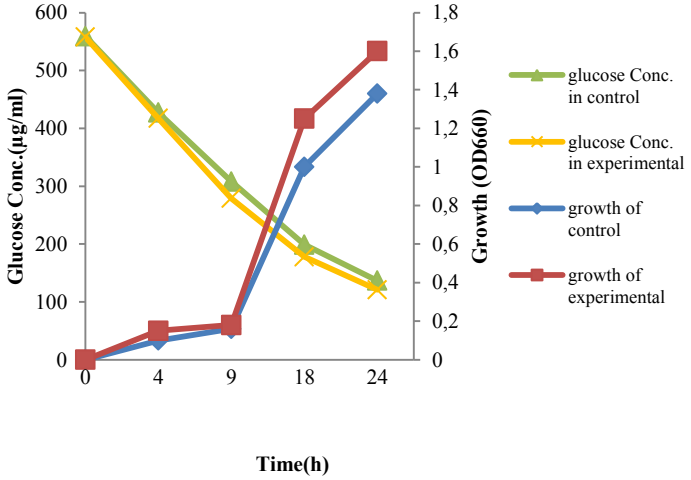

Figure 3 Faster glucose uptake by *B. parabrevis* under the influence of sound

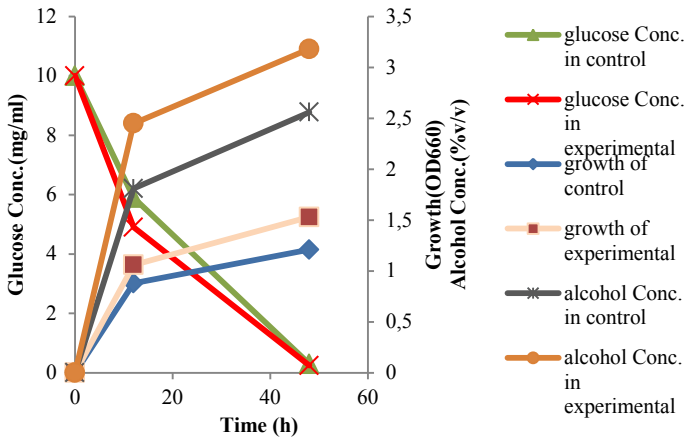

Figure 4 Faster glucose uptake and better alcohol production by *S. cerevisiae* under the influence of sound

Raag Piloo-Teentaal

Results regarding the influence of sound-II on test organisms are presented in Table 3. *S. marcescens* suffered a marginal decrease in growth and a significantly heavy decrease in pigment (prodigiosin) production owing to sound treatment. Another pigmented organism, *C. violaceum,* used in this study also suffered a decrease in growth as well as pigment (violacein) production, when incubated in presence of sound. In contrast to both these pigmented gram-negative bacteria, the third pigmented gram-negative bacterium (*X. campestris*) used in this study exhibited higher growth and even higher EPS production under the influence of sound. *C. violaceium* responded differently to both the music patterns, positively to sound-I, and negatively to sound-II; whereas remaining two gram-negative bacteria employed in this study responded similarly to both the test music patterns.

Table 3 Effect of *raag Piloo* on test organisms

Organism	**Growth (OD_{660})**			**Prodigiosin (OD_{535})**			**Prodigiosin unit (OD_{535}/OD_{660})**		
	Control (Mean±SD)	Experimental (Mean±SD)	% change	Control (Mean±SD)	Experimental (Mean±SD)	% Change	Control	Experimental	%Change
S. marcescens	0.67±0.001	0.62±0.006	-7.46**	0.42±0.004	0.16±0.007	-61.9**	0.62	0.25	-59.67
	Growth (OD_{660})			**Violacein production (OD_{585})**			**Violacein unit(OD_{585}/OD_{660})**		
	Control (Mean±SD)	Experimental (Mean±SD)	% Change	Control (Mean±SD)	Experimental (Mean±SD)	% Change	Control	Experimental	% Change
C. violaceum	1.24±0.04	0.94±0.01	-24.19**	1.29±0.10	0.77±0.04	-40.31*	1.04	0.81	-22.11
	Growth (OD_{660})			**EPS Production (g/L)**			**EPS Production per unit OD (g/L)**		
	Control (Mean±SD)	Experimental (Mean±SD)	% Change	Control (Mean±SD)	Experimental (Mean±SD)	% Change	Control	Experimental	% Change
X. campestris	1.18±0.03	1.29±0.03	9.32*	3.73±0.05	4.80±0.10	28.68**	3.16	3.72	17.72
	Growth (OD_{660}) (1:1 dilution)			**Alcohol production (% v/v)**			**Alcohol production per unit OD (% v/v)**		
	Control (Mean±SD)	Experimental (Mean±SD)	% Change	Control (Mean±SD)	Experimental (Mean±SD)	% Change	Control	Experimental	%Change
S. cerevisiae	1.28±0.01	1.34±0.0009	4.68*	1.62±0.00	1.82±0.04	12.34**	1.26	1.36	7.93
	Growth (OD_{660})(1:1 dilution)			**pH**					
	Control (Mean±SD)	Experimental (Mean±SD)	% Change	Control (Mean±SD)	Experimental (Mean±SD)	% Change			
L. plantarum	1.17±0.00	1.21±0.01	3.41**	4.30±0.00	4.16±0.05	-3.25*			
	Growth (OD_{660})			**IU**					
	Control (Mean±SD)	Experimental (Mean±SD)	% Change	Control (Mean±SD)	Experimental (Mean±SD)	% Change			
B. parabrevis	0.50±0.03	0.59±0.00	18.0*	0.027±0.00	0.032±0.00	15.35**			

***p≤0.01, *p≤0.05,* '-'minus sign indicates a decrease, when compared to control

Both the gram-positive test bacteria*, B. parabrevis* and *L. plantarum* grew better when subjected to sound treatment. The former could exhibit better cellulase activity in the sound stimulated condition, which might have allowed it to produce more biomass owing to better substrate (cellulose) utilization; or perhaps the higher cellulase activity might be simply resulting from increased cell number in the experimental tubes. The little (3.25%) but significant reduction in pH of the growth medium brought by *L. plantarum* might have resulted from more lactic acid produced (owing to 3.41% higher cell mass) under the influence of sound. *S. cerevisiae* responded positively to both the sound patterns; however, alcohol production per unit of growth was higher in response to sound-II.

Growth rate and metabolism of *S. cerevisiae* growing in liquid culture has been shown through metabolomic study to get affected by sonic vibration **(Aggio *et al.*, 2012)**. Sonic frequencies employed in their work were 100 Hz, and 1000 Hz, in addition to broad-band music. They have reported a reduction in biomass production upto 14%, when *S. cerevisiae* was grown in presence of music, with a simultaneous faster (12.4%) growth rate. In the present study, we found the cell density of *S. cerevisiae* culture growing in presence of music to be higher than in absence of music (Table 2-3). When growth rate was calculated using the data from which graph shown in Figure 4 is plotted, it was found to be 23.82% higher (P<0.01) in absence of music (0.0403 h^{-1}) than that (0.0307 h^{-1}) in its presence. In such another experiment performed with *B. parabrevis*, sound treatment was found to have a positive effect on cell density as well as growth rate (Figure 3). Growth rate in presence of music (0.212 h^{-1}) was 4.95% higher (P<0.01) than that (0.202 h^{-1}) in its absence.

This work involved investigation on effect of two different music patterns corresponding largely to the frequency ranges 172 - 581 Hz and 86 - 839 Hz on six different microorganisms. All the test organisms (except *S. marcescens,* and *C. violaceum* incubated with sound-II) grew better following incubation in presence of sound. Glucose uptake by *B. parabrevis* and *S. cerevisiae* was found to be faster under the influence of sound-I. Though few reports describing the effect of audible sound have accumulated in literature, this exciting topic warrants much more systematic studies, so that the molecular basis of microbial response to sound can be elucidated. Gu *et al.* **(2013)** reported sonic vibration (5000 Hz; 100 dB) to promote growth of *Escherichia coli*, along with an increased total protein content and antioxidant enzyme activity. Ability of music to increase bacterial growth and substrate utilization was reported by Pornpongmetta and Thanuttamavong **(2010)**. Ying *et al.* **(2009)** reported growth promoting effect of audible sound on *E. coli*.

Regarding the mechanism how audible sound affects the microbial growth and metabolism, not much can be commented with certainty. However, it may be postulated that sound waves while travelling through the liquid growth medium give rise to sonic vibrations, which may be sensed by the test microbial population through its mechanosensory receptors. Following this, the cell population may modulate its behavior in accordance to the magnitude and duration of the sonic vibration. Mechanosensitive ion channels are well

characterized force-sensing systems in living organisms. Piezo channels can sense stress (sonic vibrations can be viewed as a type of stress posed to the microbial cells), and their gating can be regulated by mechanical force. Upon sensing the stress created by the sound waves, these mechanosensitive channels may signal the organism for generation of an appropriate physiological response **(Martinac, 2012)**. Opening of such channels may be promoted by presence of membrane tension **(Sawada *et al.*, 2012)**, which in turn can affect movement of certain key ions across the cell membrane, ultimately resulting in an altered pattern of growth and metabolism. In bacteria, mechanosensitive channel proteins act as safety valves against osmotic shock, and in higher organisms they participate in sensing touch and sound waves **(Ward *et al.*, 2014)**. Once the biophysical mechanism regarding how force is sensed by mechanosensory channel protein and how the sensed force affects channel gating is completely understood, it will help to a good extent in explaining microbial response to sound stimuli. This is because through the process of mechnosensation, cells respond to variations in mechanical stress originating from sound or any such other environmental factor. Mechanosensitive channels are involved in regulation of the volume, morphology and migration of cells.

Different organisms may respond differently to a particular sound pattern because pressure distribution in the membrane varies with the type of membrane. The pressure profile of the membrane can be believed to be dependent on the intensity of the test sound, as well as, the inherent frequencies. A particular combination of a certain sound pattern and the test organism being studied will generate a particular pressure profile of the membrane, which is likely to affect the channel gating **(Sawada *et al.*, 2012)**.

CONCLUSION

This study demonstrates that microbial growth and metabolism do get affected, when exposed to external audible sound. It is generally well accepted that sound (in form of music or otherwise) affects higher forms of life. Research regarding its effect on microbes is still in infancy. Questions like: Do microbes respond differently to different frequencies of audible sound; why the response of one organism to a particular sound pattern differs from that of another organism to same sound pattern; whether audible sound can affect quorum sensing in microbial populations significantly, and influence their behaviour, etc. remains to be explored. Further developments in the mechanosensory biology, along with transcriptome and/or metabolomic profiling of sound stimulated cultures can provide meaningful insights in the area of cell-sound interaction. Research in this area can open a new frontier for multidisciplinary work at the interfaces of microbiology, biophysics, and acoustics. Understanding the molecular basis of metabolic and physiological responses of sound stimulated microbial cells may enable effective manipulation of cell metabolism and proliferation in fermentors.

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CHARACTERIZATION OF *PAENIBACILLUS DURUS* (PNF_{16}) A NEW ISOLATE AND ITS SYNERGISTIC INTERACTION WITH OTHER ISOLATED RHIZOBACTERIA IN PROMOTING GROWTH AND YIELD OF CHICKPEA

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ABSTRACT

Application of PGPR in crop production and protection is well known and can also contribute in reducing use of agrochemicals. However, the performance of PGPR is influenced by various biotic and abiotic factors. Isolating new efficient PGPR strain well adapted to local soil agroclimatic conditions is expected to perform with more consistency. In this study five selected rhizobacteria isolated previously were first tested *in vitro* for plant growth promoting (PGP) characteristics. One of the isolate PNF_{16} was identified as *Paenibacillus durus* by 16S rRNA gene sequence analysis. PNF_{16} alone and in combination with other rhizobacteria (*Azotobacter* (AZT_3), *Achromobacter* (PNF_{11}), *Bacillus* (Bc_1), *Pseudomonas* (Ps_5) and *Mesorhizobium* (IARI) were tested for plant growth promoting effect under pot conditions in two consecutive years. PNF_{16} was found to produce 21.7 µg ml^{-1} of indole acetic acid like substances, hydroxymate type of siderophores (Salicylate type 11 µg ml^{-1} and benzoate type 6.5 µg ml^{-1}) and solubilized phosphate (405.33 µg ml^{-1}). PNF_{16} -*Mesorhizobium* combination was found significantly better compared to other combinations for growth parameters, nodulation and yield of chickpea over control. Similar study was also performed for other tested strain. Significant increase in plant growth (32%), nodulation (43%) compared to untreated control was recorded. Co-inoculation also showed synergy and increased the number of pods per plant, 1000-grain weight, dry matter yield, grain yield and protein content by 23%, 22%, 21%, 18% and 4.4% respectively, compared to control. The results indicated the potential usefulness of PNF_{16} alone and in combinations in enhancement of nodulation and stimulation of plant growth in chickpea and adapted to soil condition of the region.

Keywords: *Paenibacillus*; *Mesorhizobium*; PGPR, plant-microbe interaction, nodulation, chickpea

INTRODUCTION

Interactions between plants and micro-organisms in the rhizosphere can clearly affect crop yields. Rhizobacteria that benefit plant growth and development are called 'PGPR'. The term 'PGPR' was introduced in 1978 by Kloepper and colleagues. Since then a large number of bacteria have been identified and reported as PGPR (*Acetobacter, Achromobacter, Arthrobacter, Azoarcus, Azospirillum, Azotobacter, Bacillus, Burkholderia, Clostridium, Enterobacter, Flavobacterium, Frankia, Hydrogenophaga, Kluyvera, Microcoleus, Phyllobacterium, Pseudomonas, Rhizobium, Serratia, Staphylococcus, Streptomyces, and Vibrio*) **(Bashan *et al.*, 2005)**. However commonly used PGPR in field application is limited to only few microorganisms. The plant growth promoting rhizobacteria may enhance plant growth either directly or indirectly. Direct mechanisms include (i) the ability to produce the plant growth regulators (indoleacetic acid, gibberellins, cytokinins and ethylene) **(Glick., 2012),** (ii) Asymbiotic N_2 fixation **(Ahmad *et al.*, 2008),** (iii) Solubilization of mineral nutrient like phosphates **(Taurian *et al.*, 2010),** Indirect mechanisms involve (i) antagonism against phytopathogens **(Gururani *et al.*, 2013),** (ii) Production of siderophores **(Haas and Défago, 2005),** (iii) Production of extra cellular cell wall degrading enzymes for phytopathogens β,1-3 glucanase **(Ribeiro and Cardoso, 2012),** Chitinase **(Ribeiro and Cardoso, 2012),** (iv) Antibiotic production **(Mazurier *et al.*, 2009)** and (v) cyanide production **(Ribeiro and Cardoso, 2012**). By modifying the microbial balance in the rhizosphere, PGPR can stimulate plant growth indirectly by inhibiting other deleterious microbes or root pathogens **(Berendsen *et al.*, 2012).** On the other hand, diazotrophs are able to decrease or prevent the deleterious effects of plant pathogens mostly through the synthesis of antibiotic and fungicidal compounds **(Mavingui and Heulin 1994; Dobbelaere *et al.*, 2003),** competition for nutrients (siderophore production) or by the induction of induced systemic resistance (ISR) against pathogens **(Timmusk and Wagner 1999; Dobbelaere *et al.*, 2003; Gururani *et al.*, 2013).**

A major problem associated with PGPBs is their inability to manifest PGP traits under natural field conditions consistently. This is mainly due to competition with native well adapted strains and specific nutrient limitation **(Vasssey, 2003).** We hypothesized that selecting a PGPB strains exhibiting multiple traits are expected to most ideal as the probability of expression of one or more PGP traits is higher. It is also expected that indigenous soil bacteria adapted to local soil and agro-climatic conditions exhibiting multiple PGP traits may be more effective under field conditions. We have screened rhizospheric soil in vicinity of Aligarh in northern India **(Ahmad *et al.*, 2006).** We found a new isolate of *Paenibacillus* sp. (PNF_{16}) which showed multiple PGP traits and characterized using 16S rRNA gene sequence analysis. The efficacy of PNF_{16} for plant growth promotion was assayed under pot experiment conditions alone and in combination with other bioinoculant such as *Bacillus* (Bc_1), *Azotobacter* (AZT_3), *Achromobacter* (PNF_{11}) and *Pseudomonas* (Ps_5) which were previously isolated in our laboratory.

MATERIALS AND METHODS

Isolation and characterization of bacterial isolates

Bacterial isolates PNF_{16} and other rhizobacteria used in this study were isolated and biochemically characterized using standard methods as described previously **(Ahmad *et al.*, 2006; 2008).**

Genetic identification of PNF_{16} by 16S rRNA partial gene sequencing

Single isolated colony PNF_{16} was inoculated in 5 ml Luria-Bertani (LB) broth and grown at 30 °C for 24 h. Cells were harvested and processed immediately for DNA isolation by standard procedure. The concentration and purity of the DNA preparation were determined by measuring optical density (OD) at 260 nm and ratio at 260/280 nm with a UV-Vis Spectrophotometer. The PCR amplification of almost full–length 16S rRNA gene was carried out with eubacterial specific

primers 16F27N (5'–CCAGAGTTTGATCMTGGCTCAG–3') and 16R1525XP (5'–TTCTGCAGTCTAGAAGGAGGTGWTCCAGGC–3') (**Pidiyar *et al.*, 2002**) in a final volume of 50µl. Briefly, the amplification reaction containing 50 ng templates DNA 25 pmole each of universal primers.0.2 mM dNTPs and 1.5 U *Taq* polymerase (Bangalore Genei, Bangalore, India) in 1X PCR buffer. The amplification reaction was carried out in Gene Amp PCR system 9700 (Applied Biosystems, USA) with the following conditions initial denaturation (2 min at 94 ^{o}C) was followed by 30 PCR cycles (94 ^{o}C for 30 s, 55 ^{o}C for 30 s, and 72 ^{o}C for 1 min) and a final extension at 72 ^{o}C for 5 min. Sequencing reactions were performed with Big Dye Terminator mix (Applied Biosystems) and samples were sequenced on 3730 DNA analyzer (Applied Biosystems). Nucleotide sequence data was deposited in the GenBank sequence database. The partial sequence were analysed with the Basic Local Alignment Search Tool (BLASTN) and compared to known bacterial sequences in NCBI GenBank using BLAST. Related sequences obtained from the database were used to construct a phylogenetic tree using MEGA4 software.

Quantification of PGP activities

Quantification of total IAA like compounds

Putative nitrogen fixer (PNF_{16}) was inoculated in nutrient medium (Yeast extract 1.5; Beef extract 1.5; Peptone 5; Sodium chloride 5, pH- 6.8) supplemented with 500 µg ml^{-1} L-tryptophan and incubated at 28 ^{o}C for 96 h. At the time interval of six hours, 5 ml of the bacterial culture was removed in sterile conditions. The cultures were centrifuged at 282 xg for 30 min. Two ml of the supernatant was mixed with 2 drops of orthophosphoric acid and 4 ml of the Salkowski's reagent. Development of pink color indicated IAA like compound production. Optical density was taken at 530 nm with the help of spectrophotometer (Spectronic 20D^{+}, Thermo Scientific, USA). This assay was carried out for ninety hours. Concentration of IAA like compounds produced by cultures was measured with the help of standard graph of IAA (Hi- media) obtained in the range of 10-100 µg ml^{-1} **(Lopper and Scroth, 1986).** Similarly quantitative estimation of IAA like compound was performed at different concentrations of tryptophan (0, 50-500 µg ml^{-1}) at 72 h of incubation.

Quantification of phosphate solubilization

The phosphate solubilization was quantified by measuring the soluble phosphate released in the culture medium, using standard method as described earlier (**Ahmad *et al*., 2008**). Briefly a 100 ml of sterile Pikovskaya's broth was separately inoculated with fully grown culture (10^{7} CFU ml^{-1}) of the selected isolates. The uninoculated medium served as a control. At different time intervals during growth, 10 ml of culture was removed from each flask and centrifuged at 3136 xg for 30 min and pH of the supernatant was also checked. Supernatant was transferred into tubes. To each sample, 10 ml chloromolybdic acid and 5 drops of chlorostannous acid was added and volume was adjusted to 50 ml with distilled water. The blue colour developed was read at 600 nm. Amount of phosphate solubilized was calculated using the calibration curve of KH_2PO_4.

Detection of siderophore

Siderophore production was detected by universal assay as described by **Schwyn and Nielands (1987)**. Briefly, first the glasswares were deferrated with 6N HCl overnight. Solution A, the dark blue solution (dye) (Chrom azurol S 60.5; Hexadecyltrimethylammonium bromide 72.9 in 100 ml water) was autoclaved. MM9 medium (Sucrose 342.3; Calcium chloride 147.0; Magnesium sulphate 246.4; Dipotassium hydrogen orthophosphate 20; Sodium chloride 0.2; Sodium molybdate 0.005) was deferrated separately in 3% 8-hydroxyquinoline for the removal of iron. The medium was separately washed with chloroform and traces of 8-hydoxyquinoline and chloroform were removed. The pH of Pipes (Piperazine-N, N'- bis[2-ethanesulfonic acid] buffer (30.24g) was adjusted at pH 6.8 by adding 50% (w/v) NaOH solution. All the three solutions A, B and C were autoclaved separately. After cooling the solution up to 50^{o} C, all the three solutions were added aseptically. The dye solution was finally added along the glassware with enough agitation to achieve mixing without generation of foam. Chrome azurol S agar plates were prepared and divided into equal sectors and spot inoculated with 10 µL (10^{7} CFU ml^{-1}) of test organism and incubated at 28 ^{o}C for 48-72 h. Development of yellow-orange halo around the growth was considered as positive for siderophore production. The yellow-orange halo was measured in mm around the colonies of bacterial isolates which were positive for the siderophore production.

Quantification of siderophore

Bacterial isolate PNF_{16} was further examined for the quantification of hydroxymate type of siderophores using the method of **Reeves *et al.*, (1983)**. Briefly, the glasswares and the medium were deferrated by 6N HCl and 3% of 8-hydroxyquinoline in $CHCl_3$. The single colony of bacterial isolate was inoculated in 5 ml deferrated nutrient broth to raise the inoculum. One ml (10^{7} CFU ml^{-1}) of bacterial culture was inoculated in 30 ml nutrient medium and incubated at 28 ^{o}C for three days. The fully grown cultures were centrifuged at 3136 xg for 20 min. The supernatant was adjusted at pH 2.0 with diluted HCl. Equal quantity of ethyl acetate was added twice in a separating funnel and separated, the resultant 60 ml ethyl acetate fraction was taken. Five ml of ethyl acetate fraction was mixed with 5 ml of Hathway's reagent. The absorbance was read at 700 nm for benzoate and at 560 nm for salicylates. The concentrations of benzoates and salicylates were calculated with the help of standard 2-3 dihydoxy benzoic acid (1-10 µg ml^{-1}) and salicylic acid (1-30 µg ml^{-1}) respectively.

Experimental design to assess inoculation response of test isolates on plant growth and yield of chickpea

The pot experiments on chickpea (*Cicer arietinum* L.) var. Avrodhi (purchased from Chola Beez Bhandar, Aligarh, India) were carried out in two consecutive years. Ten seeds of chickpea were sown in each earthen pots of 3 kg capacity (25 x 22 cm) in sandy clay loam soil (sand 667 g kg^{-1}, silt 190 g kg^{-1}, clay 143 g kg^{-1}, organic carbon 0.4%, Kjeldahl N 0.75 g kg^{-1}, Olsen P 16mg g^{-1}, Cation exchange capacity 11.7 cmol kg^{-1} and anion exchange capacity 5.1 cmol kg^{-1}, Calcium 30.45 mg l^{-1}, Magnesium 19.67 mg l^{-1}, Sodium 11.38 mg l^{-1}, Carbonate 18.45 mg l^{-1}, Bicarbonate 85.38 mg l^{-1}, Sulfate 16.18 mg l^{-1}, Chloride 26.69 mg l^{-1}, pH 7.4). Plants were thinned to three plants per pot ten days after emergence (DAE). The experiment was conducted in a complete randomized design and the pots were maintained in an open field conditions. There were eight treatments (i) Control, (ii) basal dose fertilizer, (iii) *Mesorhizobium* sp. , (iv) *Mesorhizobium* + *Azotobacter* (AZT_3), (v) *Mesorhizobium*+ *Achromobacter* (PNF_{11}), (vi) *Mesorhizobium*+ Fluorescent *Pseudomonas*(Ps_5), (vii) *Mesorhizobium* + *Bacillus* (Bc_1), (vii) *Mesorhizobium* + *Paenibacillus* (PNF_{16}) with each treatment was replicated three times and each pot contained three plants were used. Plants were watered with tap water. At each sampling time three plants were removed. The minimum dose of inorganic fertilizers was applied (Diammonium phosphate (DAP) @ 6.5x 10^{-2} g kg^{-1} to 4.5x 10^{-2} g kg^{-1} of soil).

Inoculum and plant culture

The standard bioinoculants, *Mesorhizobium* sp. (Indian Agriculture Research Institute, New Delhi, India) specific to chickpea were cultivated in 100 ml yeast extract mannitol broth (YEM) in 250 ml flask on rotary shaker at 125 rpm at 28 ^{o}C 2-3 days to achieve the cell density of 10^{7}-10^{8} cells ml^{-1}. All PGPR (*Azotobacter* sp., Fluorescent *Pseudomonas* sp., *Bacillus* sp., *Achromobacter* sp. and *Paenibacillus* sp.) strains were cultivated in 100 ml nutrient broth in 250 ml flasks to obtain maximum growth. The *Mesorhizobium* and all PGPR strains were mixed in the ratio of 1:1. Chickpea seeds were surface sterilized by soaking in sodium hypochlorite (5%) for 15 min and washed four times with sterile distilled water. The surface sterilized seeds were soaked in the cultures or consortium of cultures for two h at 28 ^{o}C. For control treatments as well as minimum dose of inorganic fertilizer (Di-Ammonium Phosphate) seeds were soaked in sterile distilled water. Ten seeds of each treatment were kept in 10 ml sterile NSS to take the CFU seed^{-1} of the inoculated bacteria. Inoculum density on seeds was determined by agitating ten seeds in 10 ml sterile NSS from each treatment and plated after serial dilution on the respective agar media. Plates were incubated at 28°C for 48 h, the number of colonies was counted, and the total population was expressed as CFU seed^{-1}.

Sampling and data collection

The sampling of chickpea was done at 60, 90 DAS and 145 DAS (harvest) was done. Root and shoot length and dry weight of the plants were recorded and analyzed at different stages of crop. Number of nodules and the fresh and dry weight of nodules were recorded at 90 DAS. At each sampling intervals, three pots i.e. nine plants were removed from each treatment at random. The following data were collected.

Leghaemoglobin content

Leghaemoglobin content was recorded at 90 DAS in chickpea, pink, and washed nodules from both crops were washed thoroughly and detached from the roots. Two hundred mg nodules were macerated with the help of mortar pestle in 3 ml sodium phosphate buffer, then filtered through two layers of cheese cloth. The nodule debris was discarded. The turbid reddish brown filtrate was clarified by centrifugation at 31xg for 30 min. The supernatant was diluted up to 6 ml with sodium phosphate buffer. Then equal volume of pyridine was added to extract. The solution became greenish yellow due to the formation of ferric hemochrome. The hemochrome was divided equally into two parts. To one portion few crystals of sodium dithionite was added to reduce the hemochrome. The mixture was stirred without aeration. Absorbance was read at 556 nm against the blank. Few crystals of potassium hexaferrocynate were added to other portion to oxidize the hemochrome and read at 539 nm **(Appleby and Bergersen, 1980).** Leghemoglobin was calculated by the following formula

Leghemoglobin concentration (mM) =$A_{556} - A_{539}$ x 2D / 23.4

Where D is the initial dilution, A is absorbance

Nitrogenase activity (Acetylene reduction assay) in nodules

Nitrogenase activity was recorded at 90 DAS in chickpea. The activity of nitrogenase was determined in the fresh samples by the method of **Hardy and workers (1966).** Nodules were incubated with acetylene for thirty minutes at 27 ^{o}C. The air (5cm^3) was removed with a syringe from each incubation period, and ethylene content was measured on a gas chromatograph (GC 5700, Nucon, New Delhi, India) equipped with 1.8 m Porapack N (80/100 mesh) column, a flame ionization detector and an integrator. Nitrogen was used as a carrier gas. The flow rate of nitrogen, hydrogen and oxygen were 0.5, 0.5 and 5 ml S^{-1}. The oven temperature was 100 ^{o}C and that of the detector was 150 ^{o}C. Ethylene identification was based on the retention time and was quantified by comparing with the standard curve drawn with pure ethylene. The nitrogenase activity was expressed as nM (nanomole of ethylene formed g^{-1} of nodule fresh mass h^{-1}).

Protein estimation

The protein content of seeds was estimated by the method as described by **Lowry *et al.* (1951).**

Yield parameters

At the time of harvesting, yield parameters including number of pods plant^{-1}, weight of seeds plant^{-1} and weight of thousand seeds were determined.

Statistical analysis

The data is pooled data of two years. The measured parameters were subjected to statistical calculation using one way analysis of variance (ANOVA) by SPSS ver.11.00. The difference among means was calculated at 5% probability level. When analysis of variance showed significant treatment effects, the least significant difference (LSD) ($P \leq 0.05$) was applied to make comparisons among means by Tukey's test.

RESULTS

The bacterial isolates used in this study were isolated previously from rhizospheric soil and characterized biochemically using standard methods as listed in Table 1.

Table 1 Origin, morphological characteristics, PGP activity and presumptive identification of selected bacterial isolates

Strain	Morphology	Spores/ cysts	Gram staining	Origin	PGPR traits	Most significant alignment	Identification method	References
Mesorhizobium sp.	Rods	–	Negative	Commercial biofertilizer for chickpea IARI, New Delhi	Symbiotic nitrogen fixer	-		
AZT_3	Oval	+	Negative	Rhizosphere *Trifolium alexandrium*	IAA, P solubilization, Siderophore, Antifungal activity, ammonia	*Azotobacter chroococcum*	Morphological, Biochemical (Bergey' manual)	**Ahmad *et al*. (2008)**
Ps_5	Rods	–	Negative	Rhizosphere *Triticum aestivum*	IAA, P solubilization, Siderophore, Antifungal activity, ammonia, HCN	*Pseudomonas fluorescens*	Morphological, Biochemical (Bergey' manual)	**Ahmad *et al*. (2008)**
Bc_1	Rods	+	Positive	Rhizosphere *Vigna mungo*	IAA, P solubilization, Siderophore, Antifungal activity, ammonia, HCN	*Bacillus* sp.	Morphological, Biochemical (Bergey' manual)	**Ahmad *et al*. (2008)**
PNF_{11}	Rods	–	Negative	Rhizosphere *Cicer arietinum*	IAA, P solubilization, Siderophore, Antifungal activity, ammonia	*Achromobacter* sp.	16S rRNA gene analysis	**Ahmad *et al*. (2006)**
PNF_{16}	Rods	–	Positive	Rhizosphere *Cicer arietinum*	IAA, P solubilization, Siderophore, Antifungal activity, ammonia	*Paenibacillus* sp.	16S rRNA gene analysis	This study

The bacterium PNF_{16} was found gram-positive, rod shaped, motile, without spores and cyst. Colonies on glucose broth (GB) medium are circular, convex, white and translucent. The nucleotide sequence of PNF_{16} was submitted to GenBank (accession number: KC477263). The analysis of 16S rRNA sequence indicated that PNF_{16} shared a maximum 99% homology with *Paenibacillus durus* (accession number: JQ436899). Further a phylogenetic tree was constructed from multiple sequence alignment of 16S rRNA gene sequence (Fig. 1).

The internal numbers indicates bootstrap values.

Assays for plant growth promotion traits

Isolate PNF_{16} belonging to putative nitrogen fixers was subjected to quantitative estimation of PGP traits. Indole-3-acetic acid (IAA) like molecules production in the presence of different concentration of tryptophan was analyzed. The isolate was found to produce maximum amount (21.7 µg ml^{-1}) of indole-3-acetic acid like molecules at 500 µg ml^{-1} of tryptophan concentration and at 72 h of incubation (Fig. 2 and 3). IAA like molecules production was increased significantly by increasing the concentration of tryptophan from 100-500 µg ml^{-1}. Isolate PNF_{16} solubilized phosphate (405.33 µg ml^{-1}) at 12th day of incubation (Fig. 4).The isolate also produced hydroxymate type of siderophores (salicylate type 11.0 µg ml^{-1} and benzoate type 6.5 µg ml^{-1}).

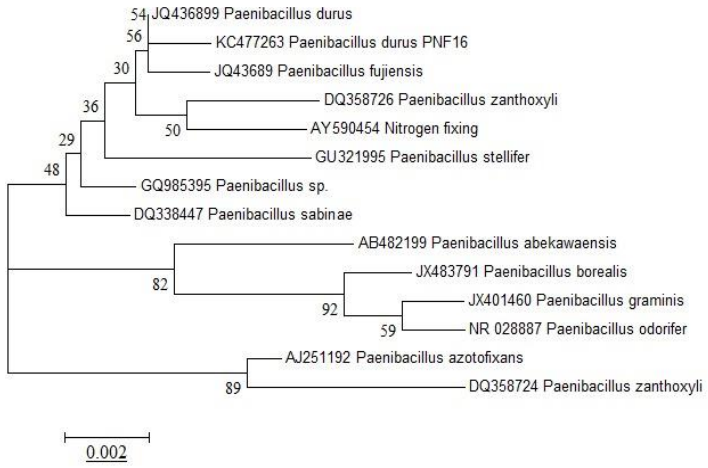

Figure 1 Phylogentic tree of PNF_{16} (GenBank accession number: KC477263) constructed using neighbor- joining method based on 16S rRNA gene sequences of strain PNF_{16} and related species.

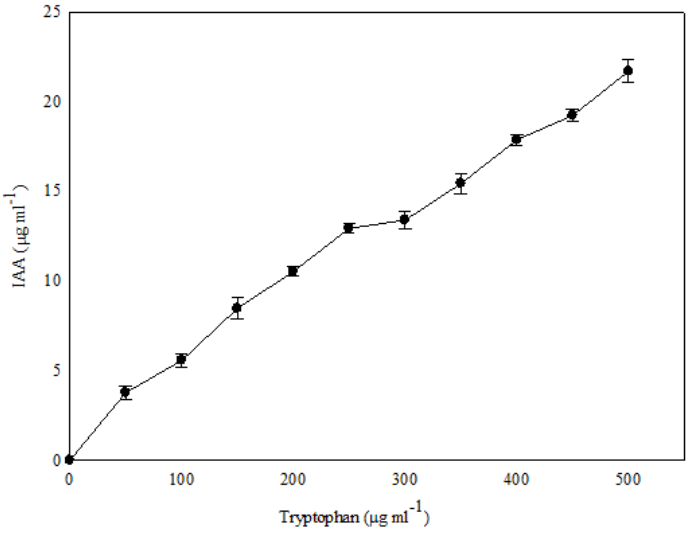

Figure 2 Tryptophan dependent IAA like substances production by PNF16 at

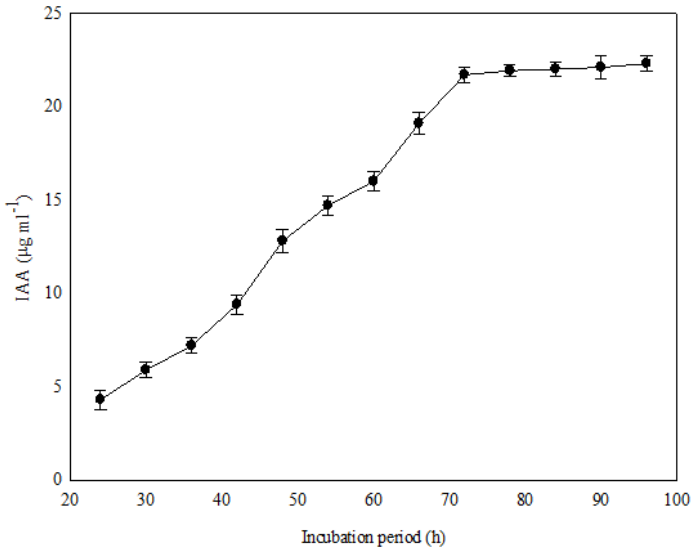

Figure 3 Time dependent production of IAA like substances by PNF_{16} at 500 72 h of incubation µg ml^{-1} tryptophan concentration.

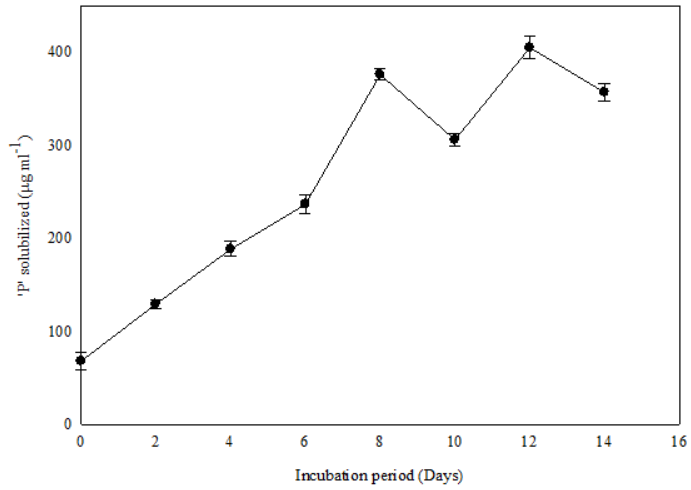

Figure 4 Phosphate solubilization by PNF_{16}

Effect of bacterial inoculation on growth, nodulation and yield of chickpea

A total of six PGPR including PNF_{16} alone and in combination were inoculated to chickpea plant by seed treatment methods under open pot conditions. Vegetative growth parameters were studied at 60, 90 days after sowing (DAS) and yield characteristics were determined at harvest (145 DAS). In general all the dual bacterial inoculation were better over control and single bacterial inoculation of *Mesorhizobium* in case of length and dry weight of plant parts i.e. root and shoot. Co-inoculation of chickpea with PNF_{16} along with *Mesorhizobium* sp. showed significant increase in root length (26%), shoot length (32%) and root and shoot dry weight (25, 27%) respectively at 90 DAS in comparison to uninoculated control (Table 2). We found that double treatment of PNF_{16} and *Mesorhizobium* was best over rest of the dual bacterial inoculation in case of vegetative parameters of chickpea.

Table 2 Effect of bacterial inoculation on growth parameters of chickpea at 90 DAS

Treatments used	Root length (cm)	Shoot length (cm)	Dry weight plant^{-1} (g)	
			Root	Shoot
Control	29.5^{c}	27^{d}	1.6^{a}	2.6^{a}
Basal dose of fertilizer	32.6^{bc}	32.6^{c}	1.7^{a}	2.8^{a}
Mesorhizobium sp.	32.7^{b}	33.2^{bc}	1.7^{a}	2.9^{a}
Mesorhizobium + *Azotobacter* (AZT_3)	32.6^{bc}	33.5^{bc}	1.7^{a}	2.9^{a}
Mesorhizobium+ *Achromobacter* (PNF_{11})	34.8^{ab}	34.6^{ab}	1.8^{a}	3.1^{a}
Mesorhizobium+ Fluorescent *Pseudomonas*(Ps_5)	33.9^{b}	33.9^{bc}	1.8^{a}	3.0^{a}
Mesorhizobium + *Bacillus* (Bc_1)	33.6^{b}	33.6^{bc}	1.7^{a}	2.9^{a}
Mesorhizobium + *Paenibacillus* (PNF_{16})	37.1^{a}	35.7^{a}	2^{a}	3.3^{a}
F value	10.982	45.152	1.27	0.728

* Column values followed by a different letters are significantly different ($P \leq 0.05$).

Nodulation parameters such as nodule number, nodule dry weight, leghaemoglobin content and nitrogenase activity was observed at 90 days after sowing (DAS). Plants treated with PNF_{16} along with *Mesorhizobium* increase the nodule number (43%) over control followed by PNF_{11} (30%), Ps_5 (27%), Bc_1 (26%) and AZT_3 (24%) (Table 3). Similarly dry weight of nodules and leghaemoglobin content showed significant increase by 42 and 63% respectively due to co-inoculation of PNF_{16} with *Mesorhizobium*. Similar trends were also observed for nitrogenase activity. *Mesorhizobium* alone inoculation showed better performance over minimum dose of chemical fertilizer as well as uninoculated control. *Mesorhizobium* and PNF_{16} were statistically significant ($P < 0.05$) over control for nodulation parameters (Table 3).

Table 3 Effect of bacterial inoculation on nodulation of chickpea at 90 DAS

Treatments used	Nodule plant^{-1}		Leghaemoglobin content[m M (g F M^{-1})]	Nitrogenase activity [nM C_2H_4 (g nodule FM.)]$^{-1}$ h^{-1}
	Number	Dry weight (g)		
Control	31.5^{d}	1.14^{d}	157.65^{e}	382.82
Basal dose of fertilizer	35^{cd}	1.185^{d}	163.05^{e}	393.09
Mesorhizobium sp.	38^{bc}	1.26^{cd}	190.35^{d}	407.9
Mesorhizobium + *Azotobacter* (AZT_3)	39.165^{bc}	1.325^{bcd}	207.1^{cd}	422.34
Mesorhizobium+ *Achromobacter* (PNF_{11})	41^{ab}	1.555^{ab}	235^{ab}	466.085
Mesorhizobium+ Fluorescent *Pseudomonas* (Ps_5)	40^{abc}	1.51^{abc}	227.1^{bc}	502.555
Mesorhizobium + *Bacillus* (Bc_1)	39.665^{abc}	1.395^{bcd}	222.05^{bc}	451.935
Mesorhizobium + *Paenibacillus* (PNF_{16})	45^{a}	1.7^{a}	256.75^{a}	436.915
F value	12.136	10.997	57.689	729.169

* Column values followed by a different letters are significantly different ($P \leq 0.05$).

The yield parameters, weight of 1000 seeds (g), number of pods plant^{-1}, number of seeds plant^{-1}, weight of seeds per plant (g) and protein content of seeds (mg g^{-1}) per plant were taken into account. The data on all yield parameters was significantly different over control. The relative inoculation responses of all four PGPR isolates are almost statistically different for yield data (Table 4). The co-inoculation increase the dry biomass by (21%), number of pods plant^{-1} (23%), number of seeds plant^{-1} (20%), weight of 100 seeds (22%), seed weight g plant^{-1} (18%) and protein content (4.4%) over control in both the year.

Table 4 Effect of bacterial inoculation on yield attributes of chickpea

Bacterial inoculations	Dry biomass (g plant^{-1})	Number plant^{-1}		Seed weight (g)		Protein content of seeds (mg g^{-1})
		Pods	Seeds	1000	(plant^{-1})	
Control	9.375 ^{d}	14.5^{c}	28.5 ^{c}	203.43	5.71	23.26 ^{e}
Basal dose of fertilizer	9.815 ^{cd}	14.665 ^{c}	30.33 ^{bc}	225.275 ^{d}	5.855 ^{e}	23.33 ^{e}
Mesorhizobium sp.	9.835 ^{cd}	15 ^{bc}	31 ^{abc}	227.58 ^{cd}	5.985 ^{de}	23.485 ^{d}
Mesorhizobium + *Azotobacter* (AZT_3)	10.41 ^{bc}	15.335 ^{abc}	31.335 ^{abc}	230.105 ^{bcd}	6.07 ^{cd}	23.58 ^{cd}
Mesorhizobium+ *Achromobacter* (PNF_{11})	10.86 ^{ab}	16.335 ^{a}	32.5 ^{ab}	239.44 ^{ab}	6.42 ^{a}	23.865 ^{a}
Mesorhizobium+ Fluorescent *Pseudomonas* (Ps_5)	10.725 ^{ab}	16.165 ^{ab}	31.5 ^{abc}	235.855 ^{bc}	6.26 ^{ab}	23.77 ^{ab}
Mesorhizobium + *Bacillus* (Bc_1)	10.595 ^{abc}	15.665 ^{ab}	31 ^{abc}	233.91^{bcd}	6.175 ^{bc}	23.66 ^{bc}
Mesorhizobium + *Paenibacillus* (PNF_{16})	11.355 ^{a}	17.83	34.165^{a}	248.495 ^{a}	6.755	24.3
F value	13.178	25.945	4.855	47.767	102.404	113.698

***** Column values followed by a different letters are significantly different ($P \leq 0.05$).

DISCUSSION

Rhizobacteria can promote plant growth through phosphate solubilization, indole and siderophore production, have a potential for use as PGP inoculants to improve crops. IAA like compounds are the most common growth regulators produced by PGPR and is known to enhance plant growth. It is now common knowledge that almost all the rhizopsheric bacteria produce growth regulating substances **(Glick, 2012).** Our isolate produced significant amount of IAA like molecules with increasing concentration of tryptophan. Varying levels of IAA like molecules production was recorded in *Paenibacillus* sp., *Bacillus* sp. and *Klebsiella* sp. was also reported by **Ji *et al.* (2014**). The availability of insoluble and fixed forms of phosphorus by bacterial inoculants play an important role in increasing soil phosphorus availability and simultaneously enhancing plant growth and crop yield **(Rodriguez and Fraga, 1999; Hameeda *et al.*, 2008)**. Bacteria belonging to genera *Paenibacillus*, *Bacillus*, *Pseudomonas* etc. are reported to solubilize the insoluble phosphate compounds and aid in plant growth (**Ahmad *et al.*, 2008; Pastor *et al.*, 2014**). Siderophores can directly stimulate the biosynthesis of other antimicrobial compounds by increasing the availability of these minerals to the bacteria, which suppresses the growth of phytopathogenic fungi and function as stress factors in inducing host resistance (**Ahmad *et al.*, 2008; Zhou *et al.*, 2012**). Our results on PGP activities are in conformation with the report of **Gururani *et al.* (2013**). The isolated rhizobacteria showing multiple PGP traits are expected to perform better on inoculation because of the probability of expression of one or other traits under natural condition. It is interesting to note that PNF_{16} showed three PGP activities such as production of indole-3-acetic like molecules, phosphate solubilization and siderophore production.

Several authors have reported the beneficial effects of N_2-fixing bacteria with other PGPR on growth and yield of legumes **(Bashan *et al.*, 1993; Bashan *et al.*, 2004).** In the present study, the symbiotic N_2-fixing *Mesorhizobium* used together with the *Paenibacillus* (PNF_{16}), *Azotobacter*, *Bacillus, Pseudomonas* stimulated growth, nodulation, and yield of chickpea. **Wani *et al.* (2007)** reported that dual bacterial inoculation of *Mesorhizobium* and phosphate solubilizing *Pseudomonas* significantly increased the plant growth of chickpea plant. Similarly, *Pseudomonas* and other PGPR exhibited enhanced nodulation, nodule dry weight and plant growth in chickpea **(Verma *et al.*, 2014). Hameeda *et al.* (2010)** have reported that the dual inoculation of *Rhizobium* with *Pseudomonas* significantly increased the nodule number, nodule weight and nitrogenase activity for chickpea under glasshouse conditions.

Stefan *et al.* (2013) reported that co-inoculation of two rhizobacterial strains significantly increased nodule number, nodule weight, total biomass and yield of runner bean compared to single strain application. Similar results were also reported by **Malik and Sindhu (2011)** using *Pseudomonas* with *Mesorhizobium* sp. that significantly improved chickpea growth and its yield components as compared with the sole application.

The results observed on the N_2 fixer, *Mesorhizobium* and PGPR in culture medium promoted to study the interaction of these organisms in soils for chickpea. Co-inoculation, increased growth and yield, compared to single inoculation, provided the plants with more balanced nutrition and improved absorption of nitrogen, phosphorus and mineral nutrients **(Sánchez *et al.*, 2014).** *Azospirillum* is also considered to be a *Rhizobium* "helper" stimulating nodulation, nodule activity and plant metabolism, all of which stimulate many plant growth variables and plant resistance to unfavorable conditions **(Bashan *et al.*, 1993; Bashan *et al.*, 2004).** In the present study when symbiotic nitrogen fixing bacteria (*Mesorhizobium* sp. for chickpea), and PGPR (*Bacillus, Azotobacter,* flourescent *Pseudomonas*, *Achromobacter* sp., and *Paenibacillus* sp. [PNF_{16}]) were used in different combinations, a high level of plant growth promotion was recorded in certain treatments with the added benefit of greater yield under unsterilized soil conditions.

Further advantages of mixed cultures over single strains have been well documented in literature for eaxmple: i) *in vitro* studies have shown that *Azospirillum* can produce more phytohormones when grown in mixed culture **(Spaepen *et al.*, 2007; Dardanelli *et al.*, 2008),** ii) mixed cultures provide conditions more suitable for nitrogen fixation than pure cultures **(Holguin and Bashan 1996; Remans *et al.*, 2008b)** and iii) mixed inoculation of biocontrol microorganisms is more efficient in controlling pathogens than the use of single strain inoculants, e.g., combinations of *Pseudomonas* with *Serratia* **(Frommel *et al.*, 1991; Hameeda *et al.*, 2010; Verma *et al.*, 2014).**

CONCLUSION

The co-inoculation of *Paenibacillus durus* (PNF_{16}) and *Mesorhizobium* sp. followed by other PGPR were found highly effective for significant enhancement in growth, nodulation and yield of chickpea. The findings of the present investigation are encouraging and needs further investigation on mechanisms of interaction in the rhizosphere as well as field performance of this new isolate *Paenibacillus durus* (PNF_{16}) which has demonstrated promising PGP activities and compatible with other tested PGPRs.

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ISOLATION AND IDENTIFICATION OF DOMINANT LACTIC ACID BACTERIA FROM *DAHI*: AN INDIGENOUS DAIRY PRODUCT OF NEPAL HIMALAYAS

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ABSTRACT

Morphological, cultural, physiological and biochemical characteristics were employed to identify dominant *Lactic acid bacteria* (LAB) isolates from 39 *dahi* (indigenous dairy product) samples collected from different districts of eastern Nepal. The isolates comprised of predominately *Lactobacillus fermentum, Lactobacillus delbrueckii* subsp. *bulgaricus, Streptococcus thermophilus, Lactococcus lactis* subspecies *cremoris, Lactococcus lactis* subsp. *lactis* biovar *diacetylactis* and *Leuconostoc mesenteroids* subsp. *mesenteroids. S. thermophilus* were found consistently in most of the samples examined. In this study, 59.38% of *Lactobacilli*, 21% of *Streptococci*, 8.2 % *Lactococci* and 11.42 % *Leuconostocs* were isolated from indigenous *dahi.* This study revealed diversity of lactic acid bacteria in Nepalese milieu having immense potential in producing qualitative fermented milk.

Keywords: Isolation, Characterization, LAB, fermented dairy product, *dahi*

INTRODUCTION

Fermentation is one of the ancient food processing and preserving techniques used all around the world. There are many indigenous fermented foods from different regions. *Dahi* or curd is one of the popular fermented dairy products from South Asia. It is prepared from boiled milk, fermented in a traditional way by natural micro flora **(Bhattarai and Das, 2013)**. Traditionally, *dahi* is consumed as a dessert or refreshing beverage along with main course in different rituals **(Kharel *et al.*, 2010)**. In Nepal, it has been consumed with boiled rice or ''*chewra*'' (rice flakes) **(Dewan and Tamang, 2007)** or use it to make as *lassi* (popular, traditional, yogurt-based drink) and *dahi-wada* (dish consisting of balls made from ground lentils, deep-fried and served in a yogurt sauce) **(Bhattarai and Das, 2013)**. Fermentating milk to *dahi* itself is a primary step for other indigenous dairy products like *nauni* (Nepalese traditional butter), *ghee* (traditional clarified butter), *mohi* (traditional butter milk) and *chhurpi* (traditional hard cheese).
As much as 45 % of total milk production is fermented into *dahi* in Eastern Nepal **(Bhattarai and Das, 2013)**. In Nepalese indigenous way, *dahi* is fermented in *theki* (a wooden utensil) made from *daar (Boehmeria rugulosa)* wood. Main purpose of *daar theki* is believed to give unique flavor in *dahi* **(Bhattarai and Das, 2013)** and might serve as natural micro flora reservoir. Flavor chemistry and microbial role are major subjects of interest in *dahi*. Microbiology and diversity have been studied by some researcher in indigenous dairy products from different parts of world. Knowledge on microbial diversity of various indigenous foods can guide us in to different arena. Typical microbial species and strains from indigenous food origin could be used to make starter culture or other industrial product. Among many important species, groups of Lactic Acid Bacteria (LAB) are vital and natural micro flora in different foods due to their unique fermentation mechanism **(Gilliland, 1990)** relating to their health and nutritional benefits **(Francois *et al.*, 2007).** LAB isolation from fermented milks have been practiced to screen desirable traits so as to obtain consistent quality, high productivity and safety **(Erkuş, 2007).**
Isolation of microbial strains from ready to eat food products helps in maintaining the food safety standards in food industries **(Nawaz and Bhattarai, 2015)**. Identification and characterization of LAB in various indigenous foods from different parts of the world have been reported, for example: *rob* of Sudan **(Abdelgadir *et al.*, 2001; Abdalla and Hussain, 2010)**, *rayeb* of Egypt **(Al Rubayyi *et al.*, 2010)**, *amasi* of Zimbabwe **(Gran *et al.*, 2003)**, *Fulani* of Burkina Faso **(Savadogo *et al.*, 2004)**, *kulenaoto* of Kenya **(Mathala *et al.*, 2004)**, *laban* of Lebanon **(Chammas *et al.*, 2006)**, *nyarmie* of Ghana **(Obodai *et al.*, 2005)**, fermented milk from the Romania **(Zamfir *et al.*, 2006)**, Tibet **(Airidengcaicike *et al.*, 2010)** and Mongolia **(Oki *et al.*, 2014)**. Very limited studies have been reported on microbial diversity and safety on Nepalese fermented milk products. This paper is therefore aimed at isolating and identifying lactic acid bacteria associated with indigenous *dahi* from eastern Nepal.

MATERIAL AND METHODS

Collection of samples

Thirty nine indigenous *dahi* samples were collected from sixteen districts of eastern Nepal. Preliminary study was done to confirm the highly dense *dahi* producing locations in different districts. No specific permissions were required for sample collection in these areas as local people were helpful and were enthusiastic to understand the quality and microbial flora of indigenous *dahi*. The samples were aseptically collected in sterile screw capped test tubes using sterile latex gloves and kept cool in ice-box until taken to the food microbiology laboratory at Central Campus of Technology, Dharan, Nepal. Samples were kept below 4°C for further use and examinations. No human or animals were used in the experiments as well as the field studies did not involve endangered or protected species.

Isolation of Lactobacilli, Lactococci, *Leuconostoc,* and Streptococci

Twenty five grams of *dahi* samples were homogenized with 225 mL Quarter Strength Ringer's solution to make an initial dilution (10^{-1}) and decimal dilution techniques were applied according to **Public Health England (2014)**. Aliquots with various dilutions amounting 0.1 mL were spread plated onto duplicate of different media plates (MRS agar, SL agar and D agar). MRS agar (Himedia, Mumbai, India) plates were incubated under anaerobic condition in an Anaerobic Gas-Pack system (Himedia, Mumbai, India) at 30°C for 48–72 h to isolate

Lactobacilli **(Badis *et al.*, 2004a)**; *Streptococcus lactis* differential agar plates (SL) (Himedia, Mumbai, India) were incubated at 37°C for 48 h to differentiate citrate utilizing and non-utilizing *Lactococci* **(Kempler, Mckay, 1980)**; MRS-vanomycin (vanomycin 20 mg.L^{-1}) plates were incubated at 30°C for 24 h to isolate *Leuconostoc* **(Mathot *et al.*, 1994)**; differential agar mediums (D) were incubated at 32°C for 48 h to differentiate *S. lactis* and *S. cremoris* **(Reddy *et al.*, 1969)** and *S. thermophilus* (ST) agar plates with added cycloheximide (100 mg.L^{-1}) for inhibition of yeast growth **(Beukes *et al.*, 2001)** were incubated at 42°C for 24-48 h to isolate Streptococci **(Atlas, 2004)**. Colonies were selected randomly from agar plate. If the plate contained less than 10 colonies, all colonies were isolated. Purity of the isolates were checked by streaking again and sub-culturing on fresh media plates, followed by microscopic examinations. Isolated strains of *Lactobacilli* and Streptococci were preserved in MRS and ST broth at - 20°C. Purified strains of *Lactococci* and *Leuconostoc* were preserved subsequently on the same media through periodic transfer.

Identification of the bacterial strains

Those preserved isolated strains were tested for gram staining, catalase production and spore formation by method from **Harrigan and McCance (1976)**. Hydrolysis of arginine, citrate utilizations, gas formations from glucose in MRS broths containing inverted Durham tubes, dextran productions from sucrose in MRS+ST agar, growths on different temperature (10, 37 and 45°C) for 5 days, resistance to 60°C for 30 min (Sherman test), growths in the presence of 4 and 6.5% (w/v) NaCl and different pH (4.5 and 6.5) and changes in turbidity of MRS broth after 24, 48 and 72 h of incubations were implicated to identify the strains **(Mayeux *et al.*, 1962; Sharpe, 1979; Samelis *et al.*, 1994;)**. Arginine MRS medium and Nessler reagent were employed to perform the hydrolysis tests as described by **Yavuzdurmaz (2007)**. Citrate utilization and colored colonies growth were observed in SL and D agars and results were interpreted according to **Reddy *et al.* (1969)** and **Kempler and Mckay (1980)**.

Sugar fermentation tests

Membrane (0.45 µm) filtered 1% (w/v) solutions of different sugars (glucose, fructose, lactose, galactose, maltose and mannitol) were deployed to study fermentation characteristics of the isolates. Nutrient broth (0.8%) with 1 mL phenol red was autoclaved at 121±1°C for 15 minutes then cooled to room temperature. Five ml of broth and 100 µL of sugars were taken into sterilized test tubes. These tubes were checked for contamination by placing at room temperature for 24 hours. After 24 hours, the purified colonies were inoculated into test tubes with specific sugar containing broth and incubated at 37°C for 48 hours. The positive test for sugar fermentation was indicated by color change from red to yellow in the test tubes as mentioned by **Mehmood *et al.* (2009)**.

RESULTS AND DISCUSSION

Isolation and characterization of thermophilic LAB

Isolation and characterization of *thermophilic* LAB is highly desirable for dairy manufacturing as the selective species are used as starter cultures. *Lactobacilli* and Streptococci isolates characterized and counted are presented in Figure 1. Mean counts of all *Lactobacilli* were 133×10^6cfu.g^{-1} representing 59.38% and Streptococci were 47×10^6cfu.g^{-1} representing 21% of total isolates from *dahi* samples. The isolated *Lactobacilli* were further characterized into different species based on their physiological and biochemical properties (Table 1). In most of our indigenous collected *dahi* samples, *Lactobacilli* were dominant *bacilli* and *S. thermophilus* were dominant *cocci*. These findings are according to previous one on Mongolian fermented dairy product where *Lactobacilli* are dominant **(Oki *et al.*, 2014)**. *S. thermophilus* plays major roles in the coagulation of milk and is responsible for the production of *dahi* and its quality. Predominant role of *Lactobacilli* and Streptococci in the indigenous sample from eastern Nepal were somewhat different from some previous results for fermented milks from different origination **(Baldorj *et al.*, 2003; Mathara *et al.*, 2004; Xiao *et al.*, 2004, Harun-ur-Rashid *et al.*, 2007, Watanabe *et al.*, 2008, Yu *et al.*, 2011).** Not always Lactobacillus and Streptococcus are dominant in all traditional fermented dairy products such as *Leuconostoc* are dominant over *Lactobacillus* South African fermented milk **(Beukes *et al.*, 2001)**. On the other hand, *S. thermophilus* are important in Greek feta cheese **(Manolopoulou *et al.*, 2003)**, in traditional Ugandan beverage *bushera* **(Muyanja *et al.*, 2003)** and commercial Nigerian bottled yoghurt **(Omafuvbe *et al.*, 2011)**. While, *dahi* from Bangladesh could be considered different in terms of dominant *S. bovis* instead of *S. thermophilus* **(Harun-ur-Rashid *et al.*, 2007)** than *dahi* from eastern Nepal.

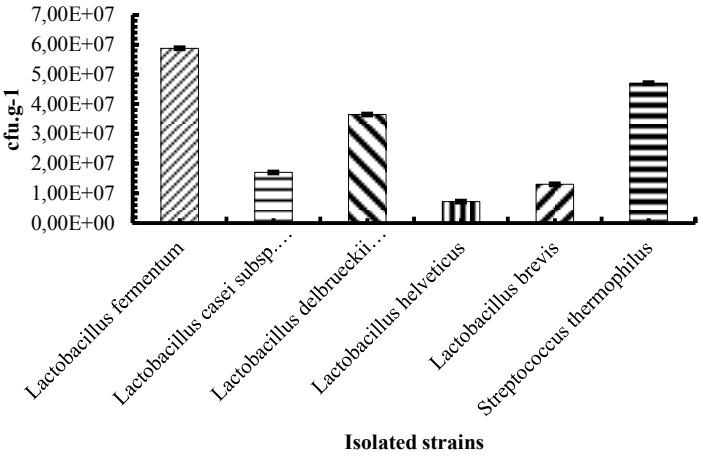

Figure 1 Mean counts (cfu.g^{-1}) of isolated strains of *thermophilic* lab found in *dahi* samples obtained from different districts of eastern Nepal

LAB are omnipresent in dairy products. Physiological and biochemical characteristics of isolated LAB strains from *dahi* of eastern Nepal were done (Table 1). Presence of different *Lactobacilli* species in *dahi* samples from Eastern Nepal can be explained by presence of these bacteria as natural micro flora in raw milk sources. To produce different dairy product, it is important to use specific fermentation methodology along with natural micro flora and desired fastidious species such as *L. delbrueckii* subsp. *bulgaricus*, *L. helveticus* etc. *L .helveticus* has been major contributor in starter cultures of some cheese like Gruyere, Gorgonzola and Mozzarella **(Tserovska *et al.*, 2002)**. This can be explained as the different strains are indigenous micro biota to milk from different animal sources. Raw milk from four races of Algerian goat was abundant of *L. helveticus* and *L. delbrueckii* subsp. *bulgaricus* **(Badis *et al.*, 2004b).** Other *Lactobacilli* species like *L. plantarum, L. brevis* and *L. delbrueckii* subsp. *bulgaricus* are isolated from *bushera* **(Muyanja *et al.*, 2003)** and *L. delbrueckii* subsp. *bulgaricus* from South African traditional fermented milk **(Beukes *et al.*, 2001)**.

Table 1 Physiological and biochemical characteristic of isolated thermophilic strains from different districts of Eastern Nepal.

Characteristics Of The Strains	*L. fermentum*	*L. casei* subsp. *casei*	*L. delbrueckii* subsp. *bulgaricus*	*L. helveticus*	*L. brevis*	*S. thermophilus*
Gram Strain Reaction	+	+	+	+	+	+
Catalase Activity	-	-	-	-	-	-
Glucose Fermentation	+	+	-	+	+	-
NH_3 From Arginine	-	-	-	-	+	-
Growth At Temperature °C						
10	-	-	+	-	+	-
37	V	-	+	+	-	+
45	+	-	+	+	-	+
Growth in a Medium With NaCl, %						
4	+	+	-	+	-	-
6.5	-	-	-	+	-	-
Growth at pH						
4.5	-	+	+	+	+	-
6.5	+	+	+	-	+	+

Production Of CO_2 from Glucose	+	-	-	-	+	-
Dextran Production	-	-	-	-	-	V
Citrate Utilization	-	-	-	-	-	-
Heat Resistance at 60 ^oC for 30 min	-	-	-	+	-	+
Sugar fermentation tests						
Fructose	+	+	+	-	+	+
Galactose	+	+	-	+	+	-
Glucose	+	+	-	+	+	+
Lactose	+	+	+	+	+	+
Maltose	-	+	-	-	V	-
Sucrose	+	+	-	-	+	+
Mannitol	-	+	-	-	+	-

Legend: V=Variable.

In Tibetan traditional fermented milk 71.3% bacilli and 28.7% cocci have been screened and two species *L. fermentum* and *L. casei* are predominant along with variation between different regions **(Airidengcaicike *et al.*, 2010)**. This could be due to the fact that, micro flora in dairy products is affected by the climatic and other external factors like altitude. In cold climatic regions mesophilic organisms such as *Lactococcus* and *Leuconostoc* have been found to be dominant in fermented milk products while *thermophilic* bacteria such as *Lactobacillus* and *Streptococcus* in warm regions as explained by **Kurman (1984)**. Differences in the profile of LAB flora in our study as compared to previous findings could be attributed to variations in the specific environmental conditions found in Himalayan region specifically in eastern Nepal. The altitude is considered as one of the important factors that influenced the climate and then temperature variations in the different parts of Nepal. Eastern Nepal is divided into three zones, namely, Mechi, Koshi and Sagarmatha ranging from plain *terai* to Mt. Everest. Moreover, cold climatic zone in upper Himalayan area and hilly area and hot climatic *terai* have a significant role in the natural micro flora statistics. The environmental factors, such as dry climate, low temperature, scant oxygen, low atmospheric pressure, strong sunlight and long sunlight radiation in Himalayas may also contribute to these variations. This present findings are according to previous findings on different fermented dairy product from Mongolia **(Oki *et al.*, 2014)**, Kenya **(Mathala *et al.*, 2004)**, Nigeria **(Olasupo *et al.*, 2001)** and Bangladesh in terms of *L. fermentum*. It has also been doubted on the negative role of *L. fermentum* as weak coagulant, gas producer giving bad texture and taste in dairy products **(Harun-ur-Rashid *et al.*, 2007)**. In addition, this bacterium has been connected to many potential probiotic properties, such as acid resistance, bile salt tolerance and indigestible carbohydrate degradation **(Airidengcaicike *et al.*, 2010)**. Therefore, these strains required to be evaluated for probiotic applications and commercialization of indigenous *dahi*.

Isolation and characterization of *Lactococci*

Lactococci species contribute to more subtle aromas and flavor that distinguish the fermented dairy products. Gram positive, catalase negative and non-spore forming isolates were isolated from 39 different samples using SL and D agars. These were further characterized as mesophilic homo-fermentative cocci. The counts of these isolates were as presented in Figure 2. Total $7.282\times10^6\pm45.19$ cfu.g^{-1} of *L. lactis* subsp. *lactis* biovar. *diacetylactis,* $2.221\times10^6\pm13.84$ cfu.g^{-1} of *L. lactis* subsp. *lactis* and $9.05\times10^6\pm5.61$ cfu.g^{-1} *L. lactis* subsp. *cremoris* were isolated from *dahi* samples of eastern Nepal. These groups ADH^{-1} (arginine dihydrolase - negative arginine hydrolysis), $citrate^{-1}$ (negative citrate utilization) isolates from *dahi* sample were further identified as *L. lactis* subsp. *cremoris* and *L. lactis* subsp. *lactis* biovar. *diacetylactis* by physiological and biochemical characteristics (Table 2). Isolated LAB were spherical or ovoid in shape, non-motile and occurred in pairs.

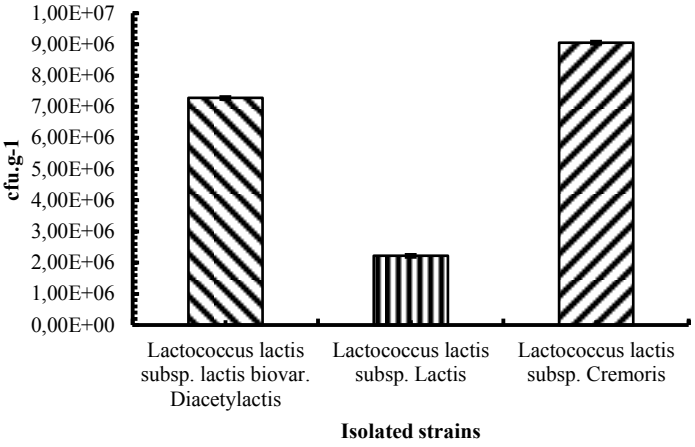

Figure 2 Mean log counts ($cfug^{-1}$) of isolated strains of *lactococccci* found in *dahi* samples obtained from different districts of eastern Nepal

These findings are accordance with some previous findings reported on isolation of *L. lactis* subsp. *lactis, L. lactis* subsp. *cremoris* from *dahi* of Himalayas **(Dewan, Tamang, 2007)** and *L. lactis* subsp. *cremoris* from Kazerun's traditional fermented yoghurt **(Azadnia *et al.*, 2011)**. Similarly, *L. lactis* are abundant in South African fermented milks **(Beukes *et al.*, 2001)** and Algerian goat milk **(Badis *et al.*, 2004b)**. On the other hand, lactic acid cocci were found to be low in number as compared to lactic acid bacilli in the mixed culture. Low numbered lacto cocci than lacto bacilli might be due to inability to cope with bacilli in *dahi* fermentation as explained by **Azadnia *et al.* (2011)**. The present study was executed to design the production of LAB starter culture. Activities of *Lactococcus* spp. are found in such a way that it might be possible to produce *dahi* consistently in quality from each batch, with its unique typical indigenous texture and flavor. In future, it might lead through the genetic characterization and selection of the most desirable strains giving highly pure commercialized strain to further elaborate the industrialization of local product.

Table 2 Physiological and biochemical characteristic of isolated *Lactococci* strains from different districts of Eastern Nepal.

Characteristice Of The Strains	Results for *L. lactis* subsp. *lactis* biovar. *Diacetylactis*	Results for *L. lactis* subsp. *lactis*	Results for *L. lactis* subsp. *cremoris*
Gram Strain Reaction	+	+	+
Catalase Activity	-	-	-
Glucose Fermentation	+	+	+
NH_3 From Arginine	+	+/-	-
Growth At Temperature °C			
10	+	+	+
37	-	-	-
45	-	-	-
Growth in a Medium With NaCl, %			
4	-	-	-
6.5	-	-	-
Growth at pH			
4.5	-	-	-

6.5	+	+	+
Production Of CO_2 from Glucose	-	-	-
Dextran Production	V	V	V
Citrate Utilization	+	-	-
Sugar fermentation tests			
Fructose	-	-	-
Galactose	-	+	-
Glucose	+	+	+
Lactose	+	+	+
Maltose	-	+	-
Mannitol	-	-	-

Legend: V=Variable.

Isolates were divided into two subgroups and a biovar. on the basis of growth pattern in SL and D agar. In SL agar, two different colored colonies were obtained. One colony was white and other Prussian blue. Totally white colonies in 48 h were identified as *S. lactis* and *S. cremoris*. Prussian blue colonies were identified as *S. lactis* subsp. *lactis* biovar. *diacetylactis*. In D agar, three different colored colonies were obtained. First types were white colonies, second yellow with yellow zones and last ones were Prussian blue. White colonies were identified as *S. lactis*, yellow colonies as *S. cremoris* and Prussian blue as *S. lactis* biovar. *diacetylactis*. The former produced small yellow colonies with yellow zones and the latter produced larger white colonies with no surrounding zones on the finalized medium. It has been justified by evident coloration in extended incubation time as explained in one of the previous studies by **Reddy *et al.* (1969)**. Occasional variability in the shade or amount of blue color on colonies was recorded with some *S. diacetylactis* strains. This might be due to natural variability on citrate transportation. However, all citrate-positive colonies were blue or large blue centered. Rapid citrate accumulation could be the reason for blue coloration of colonies and this citrate might be utilized by *S. diacetilactis*.

Isolation and characterization of *Leuconostoc*

The presence of *leuconostoc* species in fermented milk is highly desirable as it would relate to the flavorful fermented products. All grams positive, catalase negative and non-spore forming isolates were further characterized as mesophilic hetero-fermentative cocci using MRS-vancomycin media. The count of these isolates is shown in figure 3. A total of $5.789\times10^6\pm$ 14.12 cfu.g^{-1} of *Leuconostoc mesenteroides* subsp. *cremoris,* and $21.99\times10^6\pm25.70$ cfu.g^{-1} of *L. mesenteroides* subsp. *mesenteroids* were isolated from indigenous *dahi*. These bacteria represented a reduced fermentative profile, unable to hydrolyze arginine, producing gas from glucose with citrate +/- and dextran negative reactions. Physiological and biochemical characteristic of isolated strains from *dahi* samples were tested and results are presented in table 3.
Mean counts (cfug^{-1}) of isolated strains found in *dahi* samples obtained from different districts of eastern Nepal.

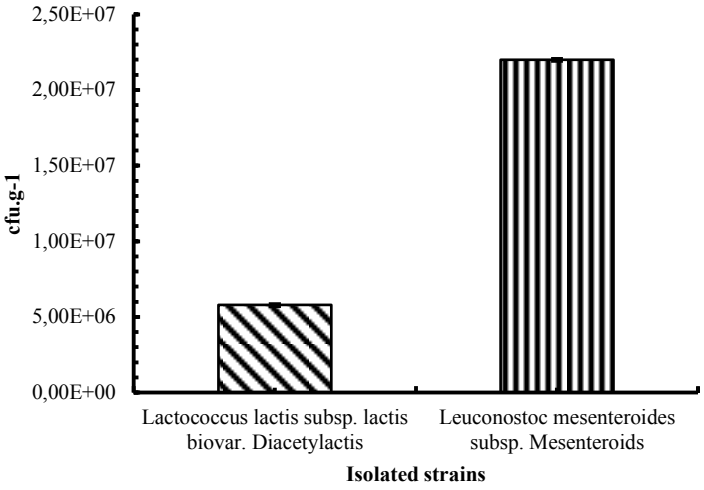

Figure 3 Mean counts (cfug^{-1}) of isolated strains of *leuconostoc* found in *dahi* samples obtained from different districts of eastern Nepal

These micro-aerophilic organisms were also characterized by the fermentation metabolism of fructose, galactose, glucose, lactose, maltose, sucrose and mannitol. *L. mesenteroides* subsp .*cremoris* was fructose, maltose and mannitol negative whereas *L. mesenteroides* subsp. *mesenteroids* were negative to mannitol only. The former utilized citrate while the latter did not (Table 3). *L. cremoris* and *L. mesenteroides* are Arginine hydrolyser and these might be present in *dahi*. Arginine hydrolysis and citrate utilization methods were tested to differentiate *L. mesenteroides* and *L. cremoris*. Arabinose fermentation was also implemented to confirm species. *L. mesenteroides* subsp. *cremoris* and *L. mesenteroides* subsp. *mesenteroids* were isolated from 39 samples but low in counts. Low numbers of these lactic acid cocci might be due to their inability to compete over lactic acid bacilli in mixed cultures. The low percentage of *Leuconostoc* strains isolated from indigenous *dahi* samples could partly be explained by their complex nutritional requirements and lower adaptation **(Azadnia *et al.*, 2011)** to dairy products. *Leuconostoc* plays important role in flavor development in dairy products **(Mataragas *et al.*, 2004)** and unique flavor in *dahi* might be attributed to this micro flora in some instances but need to do further detail study on flavor development aspects of this indigenous dairy products from Himalayas.

Table 3 Physiological and biochemical characteristic of isolated *Leuconostoc* strains from different districts of Eastern Nepal. V=Variable.

Characteristice Of The Strains	Results for *L. mesenteroides* subsp. *cremoris*	Results for *L. mesenteroides* subsp. *mesenteroids*
Gram Strain Reaction	+	+
Catalase Activity	-	-
Glucose Fermentation	+	+
NH_3 From Arginine	-	-
Growth At Temperature °C		
10	+	+
37	V	+
45	-	-
Growth in a Medium With NaCl, %		
4	-	-
6.5	V	-
Growth at pH		
4.5	-	-
6.5	+	+

Production Of CO_2 from Glucose	+	+
Dextran Production	-	V
Citrate Utilization	+	-
Heat Resistance at 60 ^oC for 30 min	-	-
Sugar fermentation tests		
Fructose	-	+
Galactose	+	+
Glucose	+	+
Lactose	+	+
Maltose	-	+
Sucrose	V	+
Mannitol	-	-

Legend: V=Variable.

Presence of *L. mesentereroides* in *dahi* samples in such numbers is somehow accordance with previous finding in indigenous fermented dairy products from Kenya **(Mathara *et al.*, 2004)**, Tibet of China **(Airidengcaicike *et al.*, 2010)**, South Africa **(Beukes *et al.*, 2001)**, Bangladesh **(Harun-ur-Rashid *et al.*, 2007)**, Uganda **(Muyanja *et al.*, 2003)**, Sudan **(Ali, 2011)**. In this context, we can say that the *L. mesenteroids* are present in most indigenous milk derived products, either from Himalayas or other part of the world.

Total LAB count in indigenous *dahi* of eastern Nepal

The total LAB counts in indigenous *dahi* of eastern Nepal were found in the range from 221×10^6 to 225×10^6cfu.g^{-1}. Mean LAB counts were $224\pm1.49\times10^6$cfu.g^{-1}. One of the previous studies reported LAB counts range between $132x10^6$ and $246x10^6$cfu.mL^{-1} **(Gandhi and Natrajan, 2010)** in *dahi* of Indian origination. In this study, 59.38% of *Lactobacilli*, 21% of Streptococci, 8.2 % *Lactococci* and 11.42 % *Leuconostocs* were isolated from indigenous *dahi* of eastern Nepal (Figure 4). The population and microbial flora of LAB might vary according to climatic condition of different regions. It is obvious that, there is high climatic and temperature variation between Himalayan area of eastern Nepal and southern hot climatic Indian terrain. In this scenario, our finding justifies the differences in microbiology of *dahi* from southern India and eastern Himalayan Nepal. Ultimately flavor dynamics, as flavor is important functions of microbes.

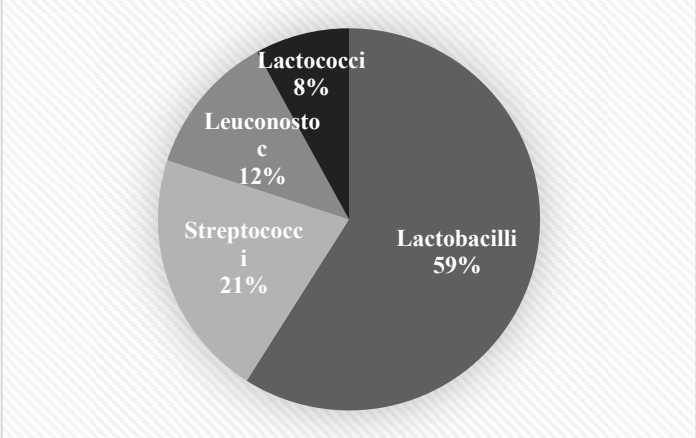

Figure 4 Distribution of LAB in indigenous *dahi* of eastern Nepal

CONCLUSION

This work showed a clear picture of microbial diversity and density in indigenous *dahi* of Nepal that might largely contribute to its typical texture and flavor. Dominant LAB comprising of *Lactobacilli*, Streptococci, *Lactococci* and *Leuconostocs* were successfully isolated from indigenous *dahi*. The wide diversity in microbial community could be attributed to variations in the specific environmental conditions found in Nepal as well as the manufacturing processes for indigenous *dahi.* Selection, propagation and preservation of the best performing strain to make starter culture could be done in future through genetic characterization. Further study on the volatile flavor compounds-matrix interactions, flavor release mechanisms, synergistic effect of flavor compounds and correlating these compounds to sensory attributes of indigenous *dahi* could be done. The profiles of volatile flavor compounds and unique role of indigenous fermentation strategy practiced could be elaborated in future.

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STUDIES ON THE OCCURRENCE OF *COXIELLA BURNETII* INFECTION IN TICKS IN SELECTED EASTERN AND CENTRAL REGIONS OF POLAND

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ABSTRACT

Arthropods may play a role in the transmission of *Coxiella burnetii* as they transmit pathogen both mechanically (as flies), and actively during the host blood suction. The aim of this study was to identify the *C. burnetii* occurring in ticks harvested from both domestic and livestock animals, as well as from wildlife in randomly selected regions of Poland. The total number 1126 ticks was collected in four regions of Poland: the Masovian, Lublin, Łódź and Podkarpackie Provinces. Among ticks collected from May 2011 to August 2012, the presence of *IS1111* gene sequence was detected in 15 (1.33%) cases by real-time PCR test. Among the ticks harvested from domestic and livestock animals in 12 cases (3.1%), and in case of ticks found in open-space rodents in 3 cases (0.4%) we found the presence of marker sequences. The low percentage of *C. burnetii* infected ticks indicate a limited role of these arthropods in Q fever transmission.

Keywords: *Coxiella burnetii,* ticks, *IS1111*, real-time PCR

INTRODUCTION

In humans, the most frequent routes of infection are via inhalation (**Dutkiewicz *et al*., 2011**), direct contact of pathogen with damaged skin and mucous membrane, via the alimentary tract, and as a result of contact with *C. burnetii* infected ticks. In the latter case the infection can be transmitted during, the tick blood suction as well as by contact with its excrements. In the *Dermacentor andersoni* faeces the bacteria survive even up to 580 days (**Woldehiwet**, **2004**).
The reservoir of *C. burnetii* comprises a wide range of species, including all vertebrates, as well as parasitic invertebrate animals. Reported data described cases of *C. burnetii* isolation from samples of animals such as sheep, goats, cattle, dogs, cats and many species of wild-living vertebrates, including bears, bison, deer, boars, rabbits, hares, shrews and marsupials. Arthropods may play a significant role in pathogen transmission, as they transmit *C. burnetii* both mechanically (e.g. flies), and actively during the host blood suction, the host may be also infected through the cutaneous or inhalation contact with the parasites faeces (**Marrie, 1990; Anusz, 1995**). The role of ticks in spreading the pathogen results from theirs manner of feeding and the ability of transstadial transmission. The ability of pathogen transmission (including transovarial) has been reported in about 40 ticks species (**Mediannikov *et al*., 2010**). Since ticks can reside on at least two to three different hosts (mainly mammals) during their life-cycle, they are considered as one of the factors determining the persistence of *C. burnetii* in the environment (**Fard and Khalili**, **2011**). The infection of ticks by *C. burnetii* occurs during the blood sucking of on infected mammal or other vertebrate, whereby they become a vector of this microorganism (**Norlander, 2000**; **Bossi *et al*., 2004; Hartzell *et al*., 2008; Oyston and Davies, 2011**).
The aim of this study was to identify the *C. burnetii* occurring in ticks harvested from both domestic and livestock animals, as well as wildlife in randomly selected regions of Poland.

MATERIALS AND METHODS

Ticks collection

Ticks were collected in four regions of Poland: the Masovian Province (central region of Poland) - 27 *Ixodes ricinus*: 5 from wildlife (deer, boar), 2 from domestic animals (cats, dogs), 20 from cattle; 4 *Dermacentor reticulatus* from domestic animals, Lublin Province (eastern Poland) - 95 *I. ricinus*: 57 from wildlife, 18 from open-space rodents, 8 from cattle, 12 from domestic animals, 2 *D. reticulatus* from domestic animals, 16 *Ixodes hexagonus* from domestic animals, Łodź Province (central Poland) - 18 *I. ricinus* from domestic animals; 2 *I. hexagonus* from domestic animals, and Podkarpackie Province (south-eastern Poland, mountains and sub-mountains region) - 699 *I. ricinus*: 325 from wildlife, 75 from open-space rodents, 62 from domestic animals, 184 from cattle, 53 from goats; 5 *I. persulcatus* from wildlife; 257 *D. reticulatus* from wildlife; 1 *Dermacentor marginatus* from wildlife.
The specimens were collected from May 2011 to August 2012. A total of 1126 ticks were collected, representing the following species: *I. ricinus* (74.5%), *D. reticulatus* (23.3%), *I. persulcatus* (0.45%), *I. hexagonus* (1.66%) and *D. marginatus* (0.09%).
Ticks were removed directly from animals, 743 specimens were taken from wild animals and another 383 ticks were harvested from domestic and livestock animals.
Among the *I. ricinus* species 839 mature individuals were collected, including 707 females feeding, 120 males and 12 nymphs. From the *D. reticulatus* species 117 adult females and 146 males were caught, and also 5 females from the species *I. persulcatus*, 17 adult females and one nymph from the *I. hexagonus* species, and from the *D. marginatus* species - one adult female. Ticks were placed in sterile 2 ml tubes and stored at -20°C until further analysis.

Ticks identification and treatment, Genetic material isolation

Identification of tick species was carried out according to the identification key (**Siuda, 1993**) using a dissecting microscope at magnification range 3.5 - 90 × in the reflected artificial light, and with regard to the larvae in transmitted light.
For tick treatment, the modified method described by **Mediannikov *et al.* (2010)** was implemented. In order to eliminate the possible microbiological contamination including *Bacillus cereus* group, ticks were treated with 0.5% solution of sodium hypochlorite for 10 minutes. In the next stage, the ticks were rinsed with distilled water, dried and re-suspended in 70% ethanol for 10 minutes. Finally, the samples were rinsed with distilled water and PBS. Remains of the fluids were then removed and dry ticks were re-suspended in 0.6 ml of MEM (Minimum Essential Medium, Sigma-Aldrich, USA) without antibiotic and homogenized by Stuart®SHM-1 apparatus. The homogenate was filled up to 2 ml

with MEM. Chitin remains were precipitated and liquid layer (1 ml) was centrifuged at 25 000 × g, the pellet was re-suspended in MEM (5 ml) and then twice filtered through 0.45 µm membrane. The filtrate was used for infection the BGM cell line (African monkey green kidney, HPA Culture Collection, UK) (**Mediannikov *et al.*, 2010)**. Bacterial DNA was isolated from 1 ml of the suspension obtained after homogenization of the ticks, using Genomic AX Tissue Mini Spin Kit (A&A Biotechnology, Gdynia, Poland) according to manufacturer's recommendations.

Real-time PCR

Real-time PCR method was used for detecting the presence of insertion sequence *IS1111* (transposase gene, GenBank: M80806) (**Klee *et al.*, 2006**) using the following primers and probe sequence: *IS1111F*: 5'-GTCTTAAGGTGGGCTGCGTG-3', *IS1111R*: 5'-CCCCGAATCTCATTGATCAGC-3', *IS1111P*: 5'- FAM – AGCGAACCATTGGTATCGGACGTTT-TAMRA-TATGG-Pho-3'.
The real-time PCR reaction was performed in a capillary system in the LightCycler 2.0 (Roche, Poland) in a final reaction volume of 20 µl. The reaction mixture consisted of the LightCycler TaqMan Master Kit (Roche, Germany) - 10 × conc., primers - 0.5 µM, probe - 0.15 µM and 5µl of template DNA.
The reaction using hydrolyzing probes for *IS1111* gene sequence was carried out according to previously optimized thermal profile: 10 min. at 95°C (initial denaturation), 15 s at 95°C, 30 s at 60°C (40 cycles of amplification), 30 s at 40°C.
As a positive control DNA extracted from *C. burnetii* strain Nine Mile phase I was used, and as a negative control a reaction mixture without template DNA was used. Real-time PCR was performed for 40 cycles, and the test samples for which C_t (Cycle threshold) was lower than 36 were considered as positive. For samples for which the fluorescence signal was weak (>36 C_T), additionally a second round of real - time PCR using the same set of primers and probes was performed.

***C. burnetii* culturing**

Culturing of the BGM cell line was carried out in the presence of MEM culture medium containing: 2 mM glutamine (Sigma-Aldrich, USA), 1% Non-essential amino acids (NEAA, (Sigma-Aldrich, USA)), 10% FBS (Fetal bovine serum, Sigma-Aldrich, USA), 100 µg/ml streptomycin and 100 U/ml penicillin (Sigma-Aldrich, USA) at 37°C in the presence of 5% CO_2.
The filtrate obtained from homogenized ticks in the amount of 0.5 ml was used for infection of the BGM cell line. Culturing of the infected cell line was carried out at 37°C in the presence of 5% CO_2. No antibiotics were added to culture medium. *C. burnetii* was then recovered from the cell line, in order to do that the method using digitonin described by **Cockrell *et al.* (2008)** was employed.

RESULTS AND DISCUSSION

From 1126 ticks harvested in the selected regions of Lublin, Podkarpackie, Masovian, and Łódź Provinces, the presence of *C. burnetii*-specific *IS1111* gene sequence was found in 15 (1.33%) cases **(Tab. 1).** Among the positive samples, five were acquired in Lublin Province (4 – *I. ricinus*, 1 – *D. reticulatus*), seven in Masovian and Łodź Provinces (6 – *I. ricinus,* 1 – *I. hexagonus*) and three in Podkarpackie Province (*I. ricinus*). In case of seven tick samples, the C_T fluorescence had the value of 31, for the next two it was above 35 cycles, and for others the signal was detected in the second round of real-time PCR. Among ticks harvested from domestic and livestock animals in 12 cases (3.1%), and in case of ticks found in open-space rodents in 3 cases (0.4%) we found the presence of marker sequences.

Table 1 Summary of positive test results for the presence of *C. burnetii* infections in ticks

No.	Province	Place/District	Animal species/ source	Tick species	Description
1.	Lublin	Puławy	Dog	*I. ricinus*	A, P, F
2.			Dog	*I. ricinus*	A, P, F
3.			Cat	*I. ricinus*	A, P, F
4.			Dog	*I. ricinus*	A, P, F
5.	Lublin	Ryki	Dog	*D. reticulatus*	A, G, F
6.	Masovian	Pułtusk	Cat	*I. ricinus*	A, P, F
7.			Dog	*I. ricinus*	A, P, F
8.			Dog	*I. ricinus*	A, P, F
9.			Dog	*I. ricinus*	A, P, F
10.	Łódź	Łódź	Cat	*I. ricinus*	A, P, F
11.			Dog	*I. ricinus*	A, P, F
12.			Dog	*I. hexagonus*	A, P, F
13.	Podkarpackie	Sanok	Open-space rodent[1]	*I. ricinus*	A, P, F
14.			Open-space rodent[1]	*I. ricinus*	A, P, F
15.			Open-space rodent[2]	*I. ricinus*	A, G, F

[1] – Striped field mouse (*Apodemus agrarius*), [2] – common vole (*Microtus arvalis*), A – Adult individual, P – The individual was feeding, G – The individual was not feeding, F – Female

Additionally, the biological material isolated from ticks and cultured in BGM cell line gave positive results of amplification in two cases of *I. ricinus:* from Masovian and Lublin Provinces.
The surveillance among ticks is rarely conducted in Poland. The studies initiated by **Anusz (1990)** revealed *C. burnetii* in females *I. ricinus* (Warmian-Masurian Province, north-eastern Poland). Simultaneously **Tylewska-Wierzbanowska *et al.* (1996)** revealed 3 (0.19%) *C. burnetii* positive ticks, out of 1580 collected in various regions of Poland (former Bydgoszcz, Koszalin, Piła, Płock, Warszawa, Kielce and Tarnobrzeg Provinces). Contrarily, another group (**Niemczuk *et al.*, 2011**) carried out studies between 2009 and 2010 on a limited number of ticks in Lublin Province and yielded 33.3% positive results of *C. burnetii* genetic material. Moreover, the same researchers between 2011 – 2013 examined the *I. ricinus* collected from endemic area in south-eastern Poland. The percentage of ticks infected with *C. burnetii* was 15.9% (**Szymańska-Czerwińska *et al.*, 2013**). Supposedly, such high percentage of positive results is related to sampling the specimens from natural focus of Q fever. Studies on the presence of *C. burnetii* among ticks from other parts of the world revealed rather low percentage of infected individuals (from 0.3% to 7.7%) (**Mediannikov *et al.*, 2010; Fard and Khalili, 2011; Špitalská *et al.*, 2003; Toledo *et al.*, 2009; Hildebrandt *et al.*, 2011; Sprong *et al.*, 2012**).
In our studies only two isolates were recovered from all 15 BGM cell line cultures. Due to intracellular type of *C. burnetii* infection, the method of bacteria recovery is crucial – significant losses of material may be sustained during the isolation procedure. The digitonin, which has high affinity to PV (parasitophorous vacuole, containing cholesterol) was successfully utilized in the present study. This intracellular structure is the place of *C. burnetii* proliferation. On the other hand, bacterial coinfections (such as *Borrelia* spp.), noted mainly among samples obtained from ticks, disturbed some cell cultures and impaired *C. burnetii* recovery from the cells. Sonication method, which is an alternative option for *C. burnetii* isolation, was not used due to accompanying risk related to unintentional generation of biological aerosol (**Cockrell *et al.*, 2008**). We used multi-copy *IS1111* gene (7 to 110 per one cell of *C. burnetii)*, which is a very convenient and sensitive genetic marker as a tool for *C. burnetii* screening in ticks (**Klee *et al.*, 2006**; **Bielawska-Drózd *et al.*, 2013**).
Pathogen may be transmitted by number of ticks species, the castor bean tick (*I. ricinus*) seems to be the most epidemiologically important species in Poland (**Tylewska-Wierzbanowska *et al.*, 1996**; **Bielawska-Drózd *et al.*, 2013**). Not

only it is the most prevalent species, but also it has a significant range of hosts. This assumption was confirmed in the current study, showing that the most of infected ticks were *I. ricinus*. The meadow tick (*D. reticulatus*) may be taken into account as the second most predominant tick in the eastern Poland (**Tylewska-Wierzbanowska and Chmielewski, 2010**).

Currently, the role of ticks as vectors and reservoir of Q fever is disputable. Some researchers (**Mediannikov *et al*., 2010; Tylewska-Wierzbanowska and Chmielewski, 2010; Sprong *et al*., 2012**) claim that ticks, as vectors of *C. burnetii* do not play a significant role in disease transmission among animals and humans. Contrarily, **Toledo *et al*. (2009)** observed high number of infected individuals among *H. lusitanicum* and *D. marginatus* population, which support the hypothesis about their role in Q fever transmission. Also other authors claim that ornate sheep tick (*D. marginatus*) is the most significant vector of *C. burnetii* in the middle Europe (**Hildebrandt *et al*., 2011**). Results of the current study demonstrated a low proportion of infected ticks (1.33%), which may confirm the first thesis. Nonetheless, ticks may be an important factor in *C. burnetii* transmission among wild animals, such as rodents, lagomorphs or wild birds (**Fard and Khalili, 2011; Astobiza *et al*., 2011**).

CONCLUSION

In our research, a low percentage of *C. burnetii* infected ticks (1.33%) indicated by the probe specific real-time PCR for multi-copy insertion sequence (*IS1111*), reveals limited role of these arthropods in Q fever transmission in Poland.

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SURVIVAL OF SELECTED BACTERIA OF *ARCOBACTER* GENUS IN DISINFECTANTS AND POSSIBILITY OF ACQUIRED SECONDARY RESISTANCE TO DISINFECTANTS

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ABSTRACT

Bacteria of the genus *Arcobacter* are today feared pathogens and represent a true threat, particularly in the form of alimentary infections. This study presents information on the inhibitory concentrations of selected disinfectant agents frequently applied in the Czech Republic, and particularly in the food industry for sanitation. The results of the study indicate that total inactivation of tested arcobacters are mostly at concentration of 5.0-15.0% in case of Desprej-disinfectant, 10.0-15.0% in case of Incidur-disinfectant, respectively 1.0-15.0% Guaa Profi Pool-disinfectant. Nevertheless, it has been confirmed that there is a real risk for secondary resistance of arcobacters to emerge in relation to certain decontaminants. Through repeated passages of arcobacters in a medium with low concentration of the disinfectant, we increased their primary resistance to the Incidur disinfectant to 1.5-3.5x (depending on species or origin). In the case of Desprej, the primary resistance of arcobacters to the substance was increased to even 1.5-5x. The emergence of resistance to antimicrobial substances is a worldwide problem. Potential acquisition of resistance (secondary resistance) in case of *Arcobacter* spp. was confirmed in this study for the first time in the Czech Republic.

Keywords: *Arcobacter* spp., disinfectant, resistance, secondary resistance, inhibition

INTRODUCTION

Arcobacters were first isolated by Ellis in 1977 from aborted cattle foetuses **(Ellis *et al.*, 1977)**. The genus *Arcobacter* was established in 1991 **(Vandamme and De Ley, 1991; Vandamme *et al.*, 1992)**. These bacteria are Gram-negative, slender, spiral-shaped rods, and are classified in the family *Campylobacteraceae* along with the genus *Campylobacter* **(Snelling *et al.*, 2006)**. Arcobacters nevertheless differ from campylobacters in their growth conditions. Arcobacters grow optimally at temperatures of 15–30°C in the presence of oxygen **(Wesley *et al.*, 2000)**. In recent years, a number of new species have been classified into this genus, which currently includes 20 species. Many species have been isolated in particular from poultry **(Atabay *et al.*, 1998)**, meat, human faeces and faeces of animals suffering from gastrointestinal tract diseases and from aborted cattle foetuses (*A. butzleri*, *A. cryaerophilus*, *A. skirrowii*, *A. cibarius*, *A. thereius* and *A. trophiarum*). Recently, however, isolation from the maritime environment also has been very common and several new species of arcobacters were described from maritime and water environment (*A. marinus*, *A. molluscorum*, *A. mytili*, *A. ellisii*, *A. bivalviorum* and *A. venerupis*) **(Collado *et al.*, 2009; Levican *et al.*, 2012a,b)**. Other representatives include *A. defluvii* from sewage water, *A. nitrofigilis* and *A. halophilus* from salt marshes **(Levican *et al.*, 2012a)** and newly classified representatives *A. cloaceae*, *A. suis* **(Levican *et al.*, 2012b)**, *A. anaerophilus* **(Jyothsna *et al.*, 2013)**, *A. ebronensis* and *A. aquimarinus* **(Levican *et al.*, 2014)**.

Antiseptics and disinfectants are used extensively in modern food and veterinary production. They are an essential part of infection control practices and aid in the prevention of clinical and subclinical diseases **(McDonnell and Russell, 1999)**. Environmental surfaces, especially those in kitchen and bathrooms, frequently have been found to be contaminated with potential pathogens, including enteric Gram-negative bacteria **(Rutala *et al.*, 2000)**. Due to the pathogenicity of arcobacters, it is necessary to establish disinfection procedures correctly, especially in the food industry but also in other operations, hospitals and households. Recently, some authors have evidenced that this organisms can survive on the surfaces of equipment in industry. Food constitutes an excellent environment for the growth of microorganisms, and their adhesion to food production equipment is very dangerous and frequently described **(Khajavi *et al.*, 2007; Fazlara and Ekhtelat, 2012)**. The threat of various alimentary infections leads to more frequent use of disinfectants, which arouses fear as to the possibility for emerging bacterial resistance. Recommended measures to reduce food contamination include biosecurity and hygiene at the farm, slaughterhouse, food industry and kitchen surfaces **(McDonnell and Russell, 1999)**. A disinfectant agent is defined as a compound reducing the numbers of viable cells of a certain group of microorganisms (not spores). Disinfectants are usually applied to inanimate objects and surfaces **(McDonnell and Russell, 1999)**. There exists a whole range of various disinfectant agent groups. Disinfectants based on a content of surfactants such as quaternary ammonium compounds (QAC) are also widely used in the food industry. They are effective against a broad array of bacteria and can be applied at a wide range of temperatures **(Møretrø *et al.*, 2012)**. Frequent industrial use of QAC-based disinfectants can lead to the development of strains resistant to these substances. Resistance can be achieved by mutation, acquisition of new genetic information, by horizontal gene transfer, expression of previously silent genes, growth in a biofilm, and other **(Chapman *et al.*, 2003)**. Thus far, however, very little information on the possibility of emerging QAC resistance in Gram-negative bacteria has been published **(Sidhu *et al.*, 2002)**.

This study deals with survival of bacteria of the *Arcobacter* genus in several selected disinfectants commonly applied in the Czech Republic, and especially in food production plants and laboratories. The study also points out the real possibility for the emergence of secondary resistance in arcobacters to selected disinfectants. To our knowledge, this subject has not yet been described in literature, it would be useful also to develop the topic of secondary resistance's acquirement in other studies.

MATERIAL AND METHODS

Bacterial cultures and cultivation

The cultures *A. cryaerophilus* CCM 3934 from the Czech Collection of Microorganisms in Brno, *Arcobacter butzleri* CCUG 30484 from the Culture Collection, University of Göteborg, Göteborg, Sweden; and *Arcobacter defluvii* LMG 25694 from the Belgian Co-ordinated Collection of Microorganisms, University of Ghent, Ghent, Belgium were used. The cultures *A. butzleri* 2013/29 and *A. cryaerophilus* 2013/23 are isolates from food of animal origin and were used as wild-type strains in this study which were isolated during our previous study **(Šilha *et al.*, 2015)**. All cultures were cultivated on Mueller-Hinton agar

(MHA, Himedia, Mumbai, India) under aerobic conditions for 48 h at 30 °C and then stored at 4 °C. Bacterial suspensions were prepared at a density of ~10^8 cfu.ml^{-1} in physiological solution and subsequently, serial decimal dilutions were prepared for other experiments in physiological solution.

Applied disinfectants

Several commercial disinfectants were chosen for testing in this study. The active ingredients and the used dilution for the tested products were as follows:
Guaa-Profi Pool (Guapex, Brno, Czech Republic): non-chlorinated disinfectant for water, active ingredients – quaternary ammonium compounds (QAC), concentrations used 0.1–15%; Incidur (Ecolab Hygiene, Brno, Czech Republic): disinfectant for surfaces and areas, active ingredients – glyoxal and glutaraldehyde, concentrations used 0.1–15%;
Desprej (Bochemie Group, Bohumín, Czech Republic): disinfectant for surfaces and areas, active ingredient – quaternary ammonium compounds (QAC), ethanol, isopropanol, concentrations used 0.1–15%.
The disinfectants were stored in the dark at room temperature and prepared used dilution in sterile Mueller-Hinton broth. All products were tested within the specified use-life.

Determining survival of *Arcobacter* spp. in disinfectants

The survival of arcobacters in selected disinfectants was determined by a suspension method in tubes **(Wiegand *et al.*, 2008)**. The range tested concentrations of the disinfectants (see figures 1-3) were prepared in tubes in Mueller-Hinton (MH) broth (HiMedia, Mumbai, India) and subsequently inoculated with 1 ml of cell suspension with density 10^6 cfu.ml^{-1} (total volume of 10 ml). After stirring the volume (30 s), 100 µl was spread onto Mueller-Hinton agar and cultivated (30 °C, 48 h). Simultaneously, the survival of arcobacters after longer time exposure (30 min, 1 h, 6 h) were tested, but arcobacters were already completely inhibited. Actual density in the initial suspension was determined by the cultivation method by inoculating the pertinent dilution onto Mueller-Hinton agar (10^2 cfu.ml^{-1}). The dependence of the number of logarithm cfu.ml^{-1} and disinfectant concentrations was observed. After each concentration, it was determined whether the cells were actually dead or in viable-but-nonculturable (VBNC) form. This was done by transferring the cell suspension in brain-heart infusion (BHI) broth (Himedie, Mumbai, Indie) under aerobic conditions for 48 h at 30 °C. As a control for the cell density introduced into mixture of disinfectant and broth, processing was always determined according to description above (without effect of disinfectant). Each determination was performed in duplicate and the experiment was repeated three times. The data obtained were statistically analysed using the standard deviation of mean.

Resistance of the bacteria to selected disinfectants

The resistance of five selected arcobacters to the disinfectants Incidur and Desprej was determined. To test the potential for the emergence of secondary resistance, 100 µl of appropriate cell suspension with density 10^8 cfu.ml^{-1} was inoculated into 9.9 ml of a mixture of Mueller-Hinton broth (pH 7.4±0.1) and a particular concentration of the tested disinfectant (according to the Table 1). Actual density in the initial suspension was determined by the cultivation method by inoculating the pertinent dilution onto Mueller-Hinton agar (10^2 cfu.ml^{-1}). Cultivation was performed at 30 °C for 48 h. At the start of the experiment, the highest concentration of the disinfecting agent at which the given microorganisms can still survive (48 h) under these conditions was determined. After two-day cultivation, 100 µl of this cell suspension was subsequently transferred to another test tube with the same concentration of the disinfectant in Mueller-Hinton broth (passaging) and continued in the same way (adjusted according to **Mrozek 1967**). After a total of five passages in the same concentration of disinfectant, 100 µl of the suspension was transferred into mixture of Mueller-Hinton broth with a higher concentration of the same disinfectant and it was determined whether the microorganisms had adapted to this higher concentration (positive growth in the tube, acquired secondary resistance). Each determination was performed in duplicate and the experiment was repeated three times. The data obtained were statistically analysed using the standard deviation of mean.

RESULTS AND DISCUSSION

The survival of *Arcobacter* spp. was determined for selected chemical disinfectants commonly applied in the food industry. Knowing how to define bacterial resistance is important for practical users of disinfectants. The effectiveness of disinfectants depends, of course, on the process and means of their application **(Sidhu *et al.*, 2002)**. One of the disinfectants tested in this study was Guaa Profi Pool (Fig. 3). It can be stated that this disinfectant product has bactericidal effects already at very low concentrations in case of majority tested arcobacters. For example, *A. defluvii* LMG 25694 was already inactivated in concentration of 1.0%. We tested lower concentration in this case of high susceptibility (0.50% - log cfu 2.37) but this point is not displayed in Fig 2. A concentration of 5.0% was determined to be the inhibitory concentration for *A. cryaerophilus* CCM 3934 during immediate inoculation (exposure time 30 s). For *A. butzleri* CCUG 30484, the inhibitory concentration was higher, concretely 7.0% during the same exposure time. However, isolate from food (*A. butzleri* 2013/23) seems to be the most resistant to this disinfectant (inhibitory concentration is 15.0%). *A. cryaerophilus* CCM 3934 is obviously one of the most sensitive tested arcobacters, inhibitory concentrations in Guaa Profi Pool, Desprej and Incidur disinfectant are 5.0%, 5.0%, resp. 10.0%. Furthermore, *A. butzleri* 2013/23 (isolate from food) seems to be the most resistant of tested arcobacters to all disinfectants. The results of the survival in these disinfectants are presented in Fig 2. As no repeated growth occurred in the inhibited culture, even after further cultivation in an environment without the given substance (the cells were actually dead, not in VBNC form). Important fact is that the wild strains tested in this study were more resistant to tested disinfectants (see Fig 1-3) in comparison with strains from culture collections.

Table 1 Emergence of secondary resistance in selected strains of *Arcobacter* genus in disinfectants (n=3).

Disinfectant	Microorganism	R_p-MIC (%)	N	M	R_s-MIC (%)	Multiple increase of R_p
Incidur	*A. butzleri* CCUG 30484	0.2	5	3	0.3	**1.5**
	A. butzleri 2013/23	0.1	5	3	0.2	**2**
	A. cryaerophilus CCM 3934	0.2	5	3	0.3	**1.5**
	A. cryaerophilus 2013/29	0.2	5	1	0.25	**1.25**
	A. defluvii LMG 25694	0.1	5	5	0.35	**3.5**
Desprej	*A. butzleri* CCUG 30484	0.2	5	15	1	**5**
	A. butzleri 2013/23	0.2	5	6	0.4	**2**
	A. cryaerophilus CCM 3934	0.1	5	8	0.5	**5**
	A. cryaerophilus 2013/29	0.2	5	16	1	**5**
	A. defluvii LMG 25694	0.1	5	3	0.15	**1.5**

Legend: N - number of passages in Mueller-Hinton broth with the same concentration of the disinfectant; M - repetition number of N; R_p-MIC - minimum inhibitory concentration - primary resistance; R_s-MIC - minimum inhibitory concentration - secondary resistance

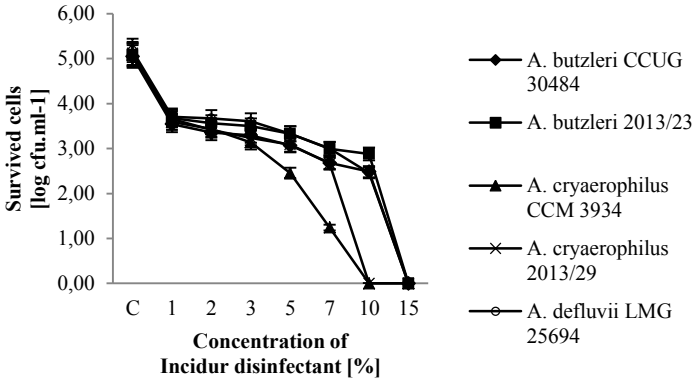

Figure 1 Survival of arcobacters in Incidur-disinfectant (exposure time 30 s) determined by a plating method. Results are expressed as $\log_{10}$ of survived cfu.ml^{-1}. Bars represent standard deviation of the mean (n=3). C – control examination (without disinfectant).

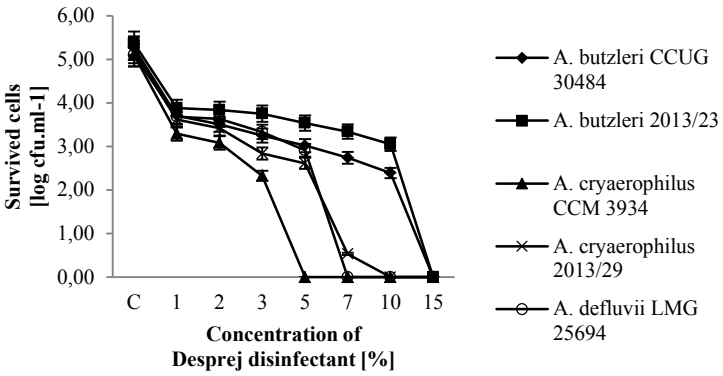

Figure 2 Survival of arcobacters in Desprej-disinfectant (exposure time 30 s) determined by a plating method. Results are expressed as $\log_{10}$ of survived cfu.ml^{-1}. Bars represent standard deviation of the mean (n=3). C – control examination (without disinfectant).

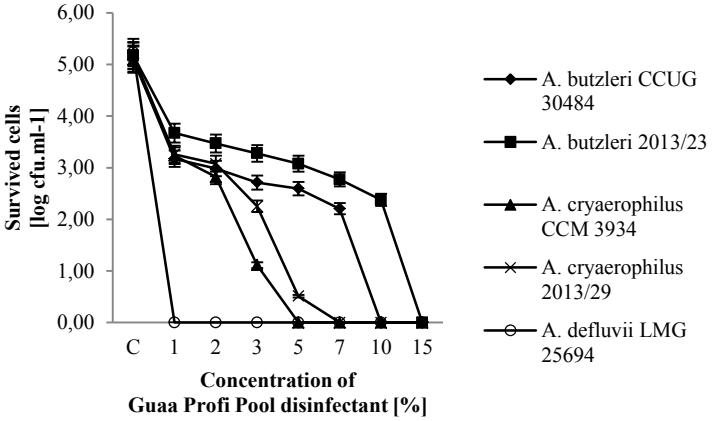

Figure 3 Survival of arcobacters in Guaa Profi Pool-disinfectant (exposure time 30 s) determined by a plating method. Results are expressed as $\log_{10}$ of survived cfu.ml^{-1}. Bars represent standard deviation of the mean (n=3). C – control examination (without disinfectant).

The results have been indicated that arcobacters are more sensitive to all tested disinfectants than are other bacteria (e.g. *Pseudomonas*, data not shown). The notable sensitivity of arcobacters to many environmental influences has already been reported **(Šilha *et al.*, 2014).** Arcobacters are closely related to *Campylobacter* spp., however, campylobacters are much more sensitive to many effects, e.g. pH values, growing in the range of 6.5–8.0, in comparison with *Arcobacter*, for which growth in the range pH 8.0–8.5 can be observed **(Cervenka, 2007)**. In our study, similar sensitivity was obtained also in experiments with tested disinfectants. The species *A. butzleri* CCUG 30484 was more resistant to the disinfectant effects. Experiments with Gram-positive bacteria such as *Staphylococcus aureus* and *Pseudomonas aeruginosa*, however, indicated their significantly higher resistance (data not displayed in the publication) in comparison with arcobacters. Arcobacters are more susceptible to many effects than Gram-positive bacteria, e.g. *S. aureus*. This is explained by the fact that the resistance mechanism is more complex in Gram-negative bacteria due to the presence of both an internal and external membrane, and also by the important role played in this respect by the presence of a lipopolysaccharide layer **(Helander *et al.*, 1997; Brula and Cooteb, 1999)**.

Disinfectants are based on various active ingredients. For example, the antimicrobial effect of substances we tested, Guaa-Profi Pool and Desprej, is based on a content of quaternary ammonium compounds. In these disinfectants, there is a greater risk for the emergence of resistance in various bacteria. This fact has already been confirmed in many studies, particularly in relation to Gram-positive bacteria of the genera *Staphylococcus*, *Listeria*, *Lactobacillus*, among others **(Sidhu *et al.*, 2002)**. Relatively little information has been published thus far about the occurrence of QAC-resistant Gram-negative bacteria in the food industry and pathogens related to food. **Willingham *et al.* (1996)** tested 350 bacterial chicken isolates in relation to their resistance to QAC, phenol and glutaraldehyde. They determined that ca 1% of strains were resistant to QAC-based disinfectants (especially *Enterobacter* and *Serratia* spp.).

One of the most important requirements for disinfectants is their safe effectiveness at low concentrations and short exposure times. However, the decreasing concentrations must not go into the range of sub-lethal concentrations and thus contribute to the potential emergence of secondary resistance in the microorganisms **(Pyle *et al.*, 1994)**.

The results of testing the potential for emergence of secondary resistance in selected bacteria of the genus *Arcobacter* confirmed that adaptations to sub-lethal concentrations of disinfectants can in fact occur. These experiments were tested in case of Desprej and Incidur-disinfectant. The third applied disinfectant in this study (Guaa Profi Pool) was so effective against arcobacters that the possibility of secondary resistance was not observed. *A. defluvii* LMG 25694 was primarily resistant to the disinfectant Incidur at 0.1% concentration (exposure time 2 d). With gradual passaging in low concentrations of the given substance at optimal cultivating temperature (as described above), the resistance was increased to a concentration of 0.35%. The resistance was thus increased to 3.5x its original, primary level of resistance. The secondary resistance in case of *A. butzleri* 2013/23 (isolates from food) was a little bit higher than in case of cultural strain of *A. butzleri* (see Table 1). However, this trend has not been proven with *A. cryaerophilus*, both, cultural strain and isolate. In another tested disinfectant, Desprej, there was a more significant emergence of secondary resistance. For the strain *A. cryaerophilus* CCM 3934, in particular, there was an increase to 5.0x the primary resistance. The results of increase from primary resistance during passaging in a disinfecting agent are presented in Table 1. Evidently, we cannot compare the results from the determination of inhibition concentrations with the results of secondary resistance. These methods are different under different conditions (inhibitory concentrations – the time of exposure 30 s; the exposure time for determination of secondary resistance 48 h – cultivation in Mueller-Hinton broth).

The discussion in case of acquired secondary resistance is complicated because of limited information about this topic in literature. To our knowledge, there are no previous data for possibility of secondary resistance in case of *Arcobacter* spp. Previously, **Růžičková and Majerníková (1999)** have already described a similar trend for the emergence of secondary resistance in Gram-negative bacteria under the effect of the material Antibacteric-P (a QAC). In that study, the resistance of *E. coli* bacteria was increased even by as much as 2,917x, and by 32x in *Citrobacter* spp.

Further studies are planned to examine resistance also in other species of arcobacters, and in relationship to several additional influences and conditions.

CONCLUSION

The genus *Arcobacter* is closely related to the well-known human pathogen *Campylobacter jejuni*. These bacteria are today dreadful pathogens and constitute a considerable threat, particularly in the form of alimentary infection. Environmental surfaces have been frequently found to be contaminated with pathogenic bacteria. The disinfection process is necessary precaution against these bacteria. Our results demonstrate that tested disinfectants have good activity against bacteria from the genus *Arcobacter* spp. The disinfectants were effective in a short time. The results indicate the wild strains of *Arcobacter* could be more resistant to disinfectant and other effects and it would be desirable to test more wild strains in future. However, the study shows the potential risk for secondary resistance of *Arcobacter* spp. to selected disinfectants. The emergence of resistance to antimicrobial substance is a worldwide problem and this topic is described in case of *Arcobacter* spp. for the first time, to our knowledge.

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