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Pitout JDD, Church DL, Gregson DB, Chow BL, McCracken M, Mulvey M, Laupland KB (2007). Molecular epidemiology of CTXM-producing Escherichia coli in the Calgary Health Region: emergence of CTX-M-15-producing isolates. Antimicrob. Agents Chemother. 51: 1281-1286.

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Full Length Research Paper

Interactive effect of Zeralenone and Fumonisin B_1 on caspace-3 kinetic activity, lactate dehydrogenase leakage, malondialdehyde levels and viability in Caco-2 cells

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Several *Fusarium* toxins are often found in combination in infested cereal grains such as peanuts, rice, wheat, maize and sorghum. Our previous study reported co-occurrence of *Fusarium* toxins fumonisin B_1 (FB_1) and Zearalenone (ZEA) in all food analyzed from Côte d'Ivoire namely rice, maize, peanut and millet. However, a few studies have been reported that address the toxicity of *Fusarium* toxins mixtures. In our preliminary previous study on combination of FB_1 and ZEA on human intestinal cell line Caco-2 results indicated that their interactive effects seemed to be antagonist effect. The aim of the present study was to investigate in the FB1+ZEA-induced antagonist effect on intestinal cells line Caco-2 using several cellular endpoints including Caspace-3 activity modulation, malonedialdehyde (MDA), cells viability as evaluated by lysosome and mitochondria integrities and cell lactate dehydrogenase (LDH) leakage. Taken together, our results were contrasted. Concerning lysosome and mitochondria integrities, combined effect of binary mycotoxins is an antagonist effect but by LDH release as a measure of cytoplasmic leakage, the interactive effect seems to be an additive effect. MDA production induced by ZEA+FB_1 was more additive effect but not synergistic effect. Caspace-3 activity modulation by ZEA+FB_1 after 6 and 24 h of incubation toxins with cells was additive effect, but after 3 h of incubation ZEA have a tendency to reduce the effect of FB_1. Our results suggest that combined effects of binary *Fusarium* toxins ZEA and FB_1 in cell line Caco-2 were unpredictable and varied according to several parameters such as the cellular endpoints and the duration of cells incubation with toxicants.

Key words: *Fusarium* toxins, interactive effect, caspace-3 kinetic activity, cells viability, malonedialdehyde (MDA), production.

INTRODUCTION

Zearalenone (ZEA) and Fumonisin B_1 (FB_1) are secondary metabolites of some toxigenic species of *Fusarium* genera. They pose a health risk not only to humans but also to livestock and, as a consequence, may cause economical losses either by unfavorable effects on domestic animals themselves or by an increased potential for health effects in human beings from consuming mycotoxin-contaminated edible animal

products. FB_1 causes liver and kidney cancer, and neural tube defects in rodents, leukoencephalomalacia in horses and pulmonary oedema in pigs (Dutton, 1996). Of major concern is the association of FB_1 with elevated incidence of human oesphageal cancer in parts of South Africa, North Eastern Iran and China, upper gastrointestinal tract cancer in Northern Italy (Chu and Li, 1994; Rheeder et al., 1992; Sydenham et al.1990) and neural tube defects in human babies (Hendricks, 1999; Marasas et al., 2004). The structures of FB1 and sphingolipids show marked similarities (Bezuidenhout et al., 1988), which may be the reason why FB_1 drastically disrupts the normal sphingolipid metabolism leading to an intracellular accumulation of sphingoid bases (mainly sphinganine relative to sphingosine), which mediate several key biological processes including inhibition of protein, DNA synthesis and apoptosis caspace-3 dependant (Abado-Bécognée et al., 1998; Soriano et al., 2005; Seefelder et al., 2003; Gopee and Sharma, 2004). In addition, FB_1 also induces lipid peroxidation in Vero cells, in primary rat hepatocytes (Abado-Bécognée et al., 1998) in C6 glioma cells (Mobio et al., 2003) and human intestinal Caco-2 cells (Kouadio et al., 2007).

On the other hand, ZEA have estrogenic and anabolic activities in several species (rodents, pigs and monkeys) (Kuiper-Goodman et al., 1987; Etienne and Dourmad, 1994), being able to cause alterations in the reproductive tract of laboratory animals (Kuiper-Goodman et al., 1987; Abid-Essefi et al., 2004). ZEA is associated with outbreaks of precocious pubertal changes in children in Puerto Rico, and has been suggested to have a possible involvement in human cervical cancer (Zinedine et al., 2007). Cellular mechanism of ZEA has been described by its high binding affinity to oestrogen receptors (Shier et al., 2001) and DNA adduct formation in female mousse tissues (Pfohl-Leszkowicz et al., 1995) and carcinogenic disorders (NTP, 1982). It has also been reported that ZEA inhibits DNA and protein synthesis and induces oxidative stress mediated cell death (Abid-Essefi et al., 2003; Ouanes et al., 2005; Kouadio et al., 2005, 2007). Consequently, the intracellular generation of reactive oxygen species (ROS) by ZEA is likely responsible for its cytotoxic and genotoxic effects (Hassen et al., 2007). ZEA causes cells death by apoptosis via caspase-independent and mitochondria/AIF-mediated pathways with a key role of activations of p53 and JNK/p38 (Yu et al., 2011).

ZEA and FB_1 could be found in the same commodities as secondary metabolites of different *Fusarium* species (Scudamore et al., 1998; Desjardins et al., 2000; Dawlatana et al., 2002). Our previous study reported co-occurrence of *Fusarium* mycotoxins FB_1 and ZEA in all food analyzed from Côte d'Ivoire such as rice, maize peanut (Sangare et al., 2006). Thus, in preliminary study on possible interactive effect of combination of FB_1 and ZEA, we reported cytotoxic effect as evaluated by cell membrane transport integrity of co-occurrence of binary FB_1 + ZEA seemed to be antagonist effect but we observed additive effect on lipid peroxydation (Kouadio at al., 2007). In order to understand the best possible interactive effect of these *Fusarium* toxins, we investigated in their combined effect regarding several cellular endpoints.

MATERIALS AND METHODS

Chemicals

ZEA and FB_1 were obtained from Sigma Chemical Company (St Louis, MO, USA) and were dissolved in ethanol/water (90:10). Dulbecco's Modified Eagle Medium (DMEM), foetal calf serum (FCS) and neutral red (NR) solution were provided from Sigma-Aldrich (Saint Quentin Falavier, France). All other chemicals used were of analytical grade and provided by Sigma-Aldrich (Saint Quentin Falavier, France).

Cell culture and treatment

Caco-2 cells, a human colon cancer cell line, were obtained from Dr. Jing Yu, Tufts School of Medicine (Medford, MA, USA) (Rousset et al., 1985). The cells were grown as monolayer culture in a high glucose concentration (4.5 g/l) DMEM medium supplemented with 10% foetal calf serum (FCS), 8 mM L-glutamine, 1% of mixture penicillin (100 IU/ml) and streptomycin (100 µg/ml) incubated at 37°C in an atmosphere of 5% CO_2—95% air mixture. For cell counting and subculture, the cells were dispersed with a solution of 0.05% trypsin and 0.02% EDTA.

Cytotoxicity assay by NR test

The NR test was performed to assess cytotoxicity, as described by Kouadio et al. (2005). Viable cells actively transport this dye across their cell membrane; therefore, after subsequent lyses absorbance can be used as a measure of cell viability. The solution stock of NR (3.3 g/l) was diluted to 1/100 in the cell culture medium and the extract solution consisted of 50% (v/v) ethanol in Milli-Q water with 1% (v/v) acetic acid. After 72 h of incubation in presence of each mycotoxin alone and their mixture (ZEA+FB1) or the vehicle, 150 ml of freshly prepared NR solution pre-warmed to 37°C was added to each well and all plates returned to the incubator at 37°C for 4 h. The cells were washed two times and 150 µl of the extract solution were added in each well and plates were shaken for 15 min. The absorbance at 540 nm was determined using a Microplate Reader DYNATECH MR 4000 manufactured by DYNATECH and provided by MICROPLATE in Business & Industrial, Healthcare, Lab & Life Science.

Cytotoxicity assay by tetrazolium-based colorimetric assay (MTT test)

MTT test was used to assess cell viability based on the capacity for

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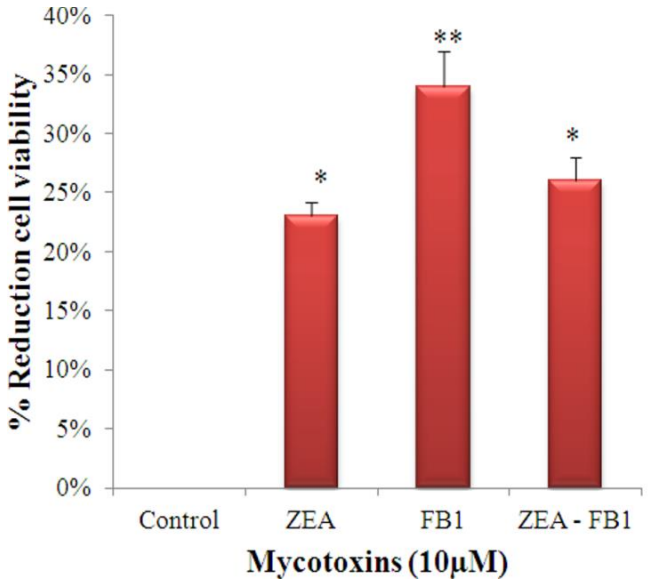

Figure 1. Cytotoxicity effect of ZEA (10 µM), FB_1 (10 µM) or their mixture on Caco-2 cells after 72 h incubation evaluated by MTT test. Results are given as mean ± SD from three independent experiments. *Different from control at $p < 0.05$ and **$p < 0.001$.

viable cells to metabolise a tetrazolium colourless salt to a blue formazan in mitochondria (Kouadio et al., 2005). After 72 h of incubation in presence of toxins ZEA, FB_1 and their mixture or the vehicle, 100 µl of 0.5% solution of thiazolyl blue tetrazolium bromide (MTT) were added to each well and 2 h later the medium was eliminated. Subsequently, 100 µl of dimethyl sulfoxide (DMSO) were added to the wells to extract the formazan formed in the viable cells. After 5 min of continuous stirring, the absorbance was determined at 540 nm using a Microplate Reader DYNATECH MR 4000. The absorbance is proportional to the number of viable cells.

Cytotoxicity assay by lactate dehydrogenase measure (LDH assay)

The LDH assay was performed to assess cytotoxicity, as described by Yusup et al. (2005). Caco-2 cells (1 x 10^5 cells/ml/well) were preincubated in 24-well multidishes for 24 h at 5% CO_2 - 95% air at 37°C. Cell viability was assessed by LDH leakage through the membrane into the medium. After 48 h of incubation in presence of mixture of toxins (ZEA and FB_1) or the vehicle, cells supernatant were used for the presence of LDH by LDH assay kit (Biomerieux, Lyon, France). In this test, three wells were used for each mycotoxin (ZEA and FB_1) and their mixture. The amount of LDH measured is related to the protein content of cellular homogenates, determined using the colorimetric method of Bradford (1976). Leakage was expressed as percentage difference from controls.

Extraction and determination of malonedialdehyde (MDA)-thiobarbituric acid (TBA) adduct

Cells (10^5 cells/ml) were cultured in 24-well multidishes (Polylabo, France) for 24 h at 37°C as described above, and then cultures were incubated in the presence of each mycotoxin alone and their mixture ZEA+FB1 for 24 h at 37°C. After this incubation, cells were trypsinised, centrifuged and resuspended in SET buffer (0.1 M NaCl, 20 mM EDTA, 50 mM Tris-HCl, pH 8.0). As described by Ennamany et al. (1995) and Abado-Bécognée et al. (1998), extraction and determination of the MDA- TBA adduct by HPLC and fluorimetric detection after extraction in n-butanol (50 µl injected for analysis) was performed. The amount of MDA measured is related to the protein content of cellular homogenates, determined using the colorimetric method of Bradford (1976).

Caspace-3 activity assay

The assay was performed according to the manufacturer's instructions, Promega, USA. After 3, 6 or 24 h of incubation at 37°C in the presence of each mycotoxin alone or their mixture ZEA+FB_1, cells (10^6 cells/ml) were disrupted by incubation ice-cold lysing buffer for 10 min and then centrifuged at 15,000 x *g* for 20 min. Supernatants (cell extracts containing caspase-3) were retrieved and 50 µl aliquots (100-200 µg total protein) along with Ac-DEVD substrate labeled with the chromophore p-nitrianiline (pNA) were added in a 96-well flat bottomed microplate. In presence of active caspase-3, cleavage and release of pNA from substrate occurs. Free pNA produced a yellow color that can be detected by spectrophotometer Microplate Reader DYNATECH MR 4000 at 405 nm. Additional controls, some free from cell lysates and others lacking substrate, were included. The results were expressed as caspase-3 specific activity (IU/mg protein).

Statistical analysis of data

The data are expressed as mean standard deviation (SD) for at least three independent determinations in triplicate or quadruplicated for each experimental point. The statistical differences between treated groups and control groups were determined by Student's t-test using SPSS 11.19 statistical software, and $p = 0.05$ was considered the limit for significance.

RESULTS AND DISCUSSION

Cytotoxicity assay by MTT test

The viability of Caco-2 cells measured with MTT tests after incubation of either individual mycotoxin FB_1 or ZEA or their mixture revealed diminishing of cell viability (Figure 1). ZEA (10 µM) reduced slightly cell viability by about 20-25%, which is lower than 10 µM FB_1 does (35-38%). Thus, ZEA appears weakly cytotoxic. Strikingly, FB_1 and ZEA mixed did not affect cell viability higher than FB_1 alone (Figure 1). Indeed, combined effect observed of these toxins is lower than expected additive effect of their mixture.

Cytotoxicity assay by NR test

The measure of Caco-2 cells viability with Neutral Red tests after incubation of mycotoxin FB_1 or ZEA alone or their mixture revealed reduction of cell viability (Figure 2). But, the induction of cell viability induced by FB_1 (10 µM) is low that is only by about 10-15%. ZEA (10 µM) reduced Caco-2 cells viability by about 23-25%.

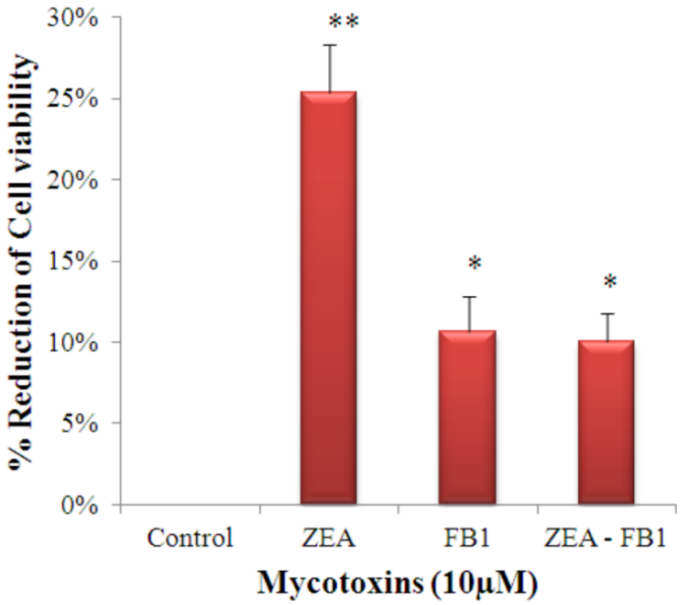

Figure 2. Cytotoxicity effect of ZEA (10 µM), FB_1 (10 µM) or their mixture on Caco-2 cells after 72 h incubation evaluated by NR test. Results are given as mean ± SD from three independent experiments.*Different from control at $p < 0.05$ and **$p < 0.001$.

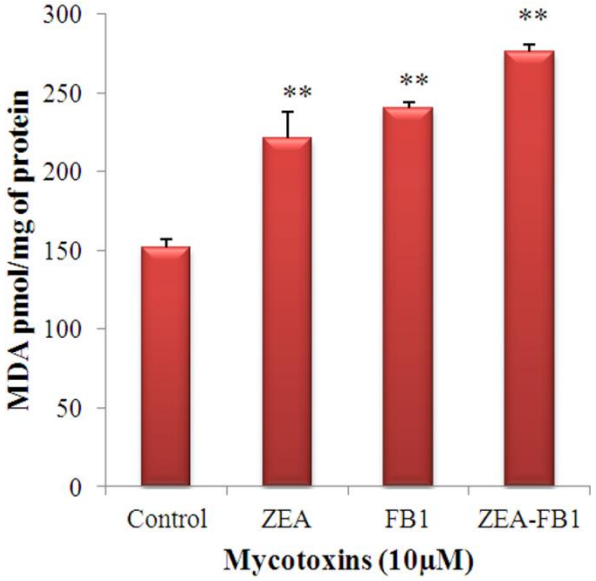

Figure 4. Lipid peroxidation as measured by MDA-TBA adduct after incubation of Caco-2 cells with ZEA (10 µM) or FB_1 (10 µM) or their mixture by 24 h. Results are given as mean ± SD from three independent experiments.*Different from control at $p<0.05$ and **$p<0.001$.

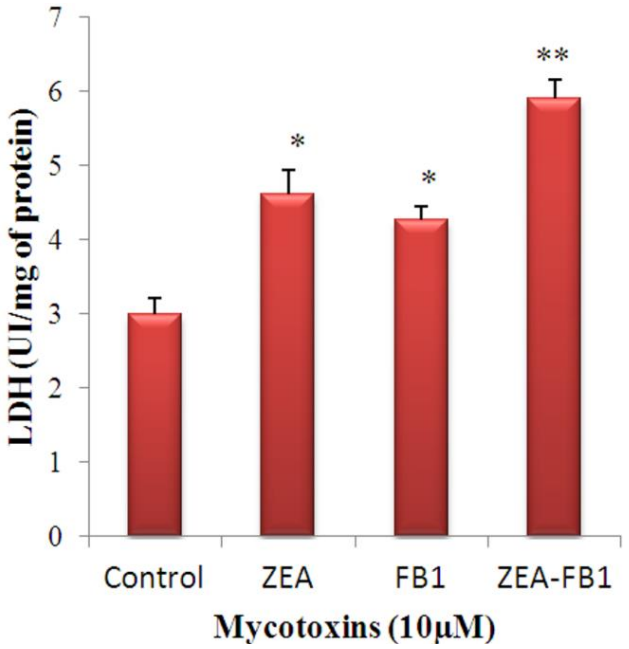

Figure 3. Increasing of LDH leakage into the cell culture medium after incubation of Caco-2 cells with ZEA (10 µM) or FB_1 (10 µM) or their mixture by 24 h. Results are given as mean ± SD from three independent experiments. *Different from control at $p<0.05$ and **$p<0.001$.

Surprisingly, addition of FB_1 to ZEA leads to reduction of cell viability similarly to those induced by FB_1 alone.

Cytotoxicity assay by LDH assay

Incubation with mycotoxin FB_1 or ZEA alone or their mixture resulted in increasing of leakage of LDH into the culture medium compared with controls (Figure 3). Effects produced by both *Fusarium* toxins were similar and the mixture induced higher increasing of leakage of LDH.

Extraction and determination of MDA- TBA adduct

ZEA and FB_1, all at a concentration of 10 µM, increased MDA production in Caco-2 cells by 33%, 36%, respectively. Their mixture also increased MDA production by about 80% (Figure 4). A rapid comparison between experimental values of MDA increases and theoretical values calculated from observed individual ones showed the addition of ZEA and FB_1 increased MDA production in a more additive way.

Caspace-3 activity assay

The modulation of activation of caspase-3 after ZEA (10 µM) or FB1 (10 µM) or their mixture exposure on Caco-2 cells was evaluated according to variable duration namely 3 h, 6 h and 24 h. As shown in Figure 5, ZEA (10 µM) or FB_1 (10 µM) or their mixture increased activity of caspase-3. But, the modulation of caspase-3 activity was

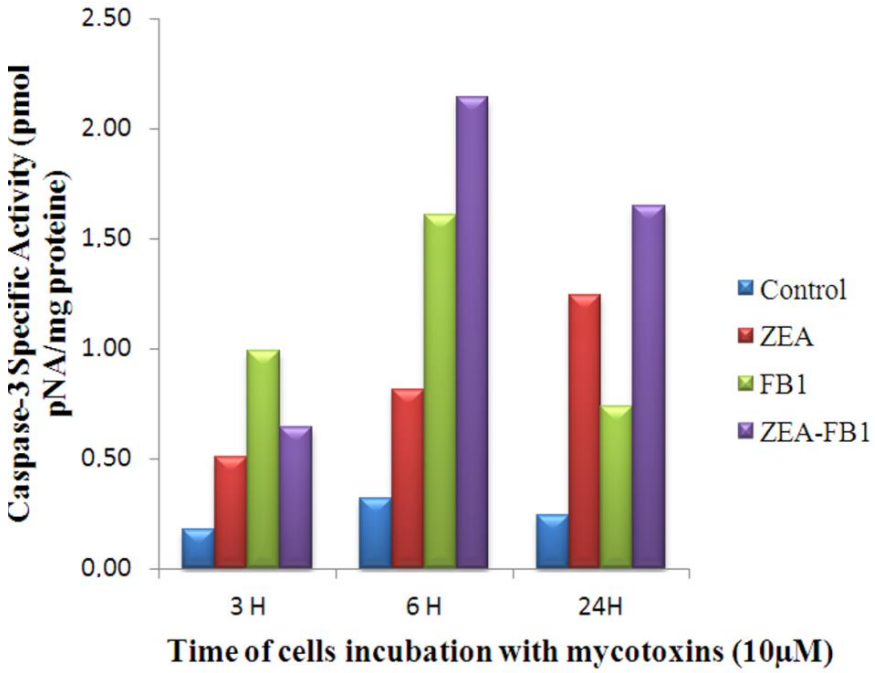

Figure 5. Activation of caspase-3 after ZEA (10 µM) or FB1 (10 µM) or their mixture exposure on Caco-2 cells for variable duration namely 3 , 6 and 24 h.

variable for the same toxin according to the duration of incubation of Caco-2 cells with toxins. The activation of caspase-3 by ZEA increased proportionally to the duration of Caco-2 cells exposure. Thus, ZEA was more potent at 24 h > 6 h > 3 h. FB_1 increased caspase-3 activity proportionally to the duration of cells exposure until 6h but the FB_1-effect induced decreased at 24 h. The mixture of ZEA and FB_1 was more potent in activation of caspase-3 at 6 h of cell exposure. In addition, the mixture of toxins leaded to additive effect on caspase-3 activation at 6 and 24 h of cells exposure in contrast to 3 h of incubation where FB_1 alone is stronger than the mixture.

DISCUSSION

Concentrations of toxins ZEA and FB_1 used in the present study were 10 µM for each mycotoxin and are those that allow distinguishing synergistic or antagonistic effects of toxins when used in mixture following our previous data (Kouadio et al., 2005; 2007). ZEA and FB_1 were tested alone at concentrations from 1 to 150 µM (Kouadio et al., 2005) and their mixture at concentrations between 4 and 40 µM (Kouadio et al., 2007). Additionally, these concentrations are the concentrations that could be reached in animals or possibly in human tissues following ingestion of 2-4 mg/kg BW of FB1 or ZEA in foods or feed (Creppy, 2002).

In our previous studies focused on interactive effect of *Fusarium* toxins ZEA, FB_1 and Deoxynivalenol (DON). It has been reported that the cytotoxic effect as evaluated by DNA synthesis and cell membrane transport integrity of combination of ZEA and FB_1 seemed to be antagonist effect in contrast to additive effect observed on lipid peroxydation and protein synthesis (Kouadio et al., 2007). In order to understand the best possible interactive effect of these *Fusarium* toxins, we investigated in their combined effect regarding several cellular specific endpoints. Thus, the binary toxins have been tested on mitochondrial succinate dehydrogenase activity and the results reveal that ZEA undoubtedly reduced FB_1 inhibition mitochondria enzyme-induced. Similarly, we have confirmed the antagonist effect of ZEA and FB_1 mixture on cell lysosome integrity (Kouadio et al., 2007). In contrast to both previous cases, the combination of ZEA and FB_1 produced an additive effect on leakage of LDH, an enzyme marker of cell membrane damage or cells necrosis (Galluzzi et al., 2009; Fotakis and Timbrell, 2006). These findings were surprising because the three cells endpoints namely lysosome integrity, mitochondrial succinate dehydrogenase activity and leakage of LDH have been always considered as makers of cells viability or cytoxicity (Babich and Borenfreund, 1987; Kouadio et al., 2005; Smith et al., 2011). Although, the differences between cell damage pathways evaluated or measured by the three assays could explain such findings. Indeed, cell membrane leakage of LDH is known as a marker of necrosis which represents passive cell death without an underlying regu-

latory mechanism or activation of executive caspases (Wyllie et al., 1980). Thus, the amount of LDH release resulted from cell disorders induced by individual effect of each mycotoxin leading necrosis cell death. In contrast, membrane transport integrity and mitochondrial succinate dehydrogenase activity represent cell specific vital functions implying regulatory or organized mechanisms which also could be targets of toxicants (Babich and Borenfreund, 1987; Kouadio et al., 2005; Smith et al., 2011). Previously, it has been reported that ZEA and FB_1 targeted the same cellular organelles namely mitochondria and/or lysosomes consequently authors have hypothesized that combinations of these toxins would lead to additive or synergistic effects (Kouadio et al., 2005). Surprisingly, in the present study, antagonist effect was observed. In fact, the concentrations of mycotoxins could modulate their interactive effect leading to unexpected findings as reported by Boeira et al. (2000) on growth of yeast. These authors reported antagonism for low concentrations and synergism for high concentrations for mixture of ZEA and DON. Concerning lipid peroxydation, association of ZEA and FB_1 leaded to additive effect as reported previously (Kouadio et al., 2007). These findings traduce the capably of ZEA and FB_1 to produce reactive oxygen species (ROS) but the nature of ROS and their mechanism of production remains unclear. However, it appears probable that FB_1 or ZEA induces ROS production in mitochondria and/or by inflammatory disorders involving TNF-α (Soriano et al., 2005; Seefelder et al., 2003; Ayed-Boussema et al., 2008; Bouaziz et al., 2008) and the intracellular generation of ROS is likely or partly responsible for their cytotoxic and genotoxic effects (Hassen et al., 2007; Mobio et al., 2003; Kouadio et al., 2007). Although, both ZEA and FB_1 target the same cellular organelle that is mitochondria, in the production of ROS (Kouadio et al., 2005), but any chemical interaction is not at stake. On the other hand, since ZEA and FB_1 have been shown to induce apoptosis caspace-3 dependent (Ayed-Boussema et al., 2008; Bouaziz et al., 2008; Soriano et al., 2005 ; Seefelder et al., 2003 ; Gopee and Sharma, 2004), we have tested their possible interactive effect on caspase-3 activity modulation. Our results have confirmed clearly modulation of caspase-3 activity induced by ZEA or FB_1 (Ayed-Boussema et al., 2008; Soriano et al., 2005). It has been reported that FB_1 modules caspase-3 activity by several pathways involving mitochondria and disturbs cytochrome-c release and cell membrane TNF-R1 receptor activation by TNF-α (Di Pietro et al., 2005). Concerning ZEA, studies reported ZEA induced apoptosis caspase-3 dependence or not (Bouaziz et al., 2008; Yu et al., 2011) and apoptosis caspase independence induced by ZEA could be related to apoptosis-inducing factor-mediated and ROS-dependent pathways, in which p53 and JNK/p38 MAPK play crucial roles as upstream effectors (Yu et al., 2011). In the present study, FB_1 has been showed to induce early apoptosis caspase-3 dependent in contrast to ZEA which was found very potent later until after 24 h of cells incubation. In contrast to FB_1, ZEA could module caspase-3 activity by long processes. The mixture of toxins led to additive effect on caspase-3 activation at 6 and 24 h of cells exposure in contrast to 3 of incubation where FB_1 alone is stronger than the mixture. In fact, ZEA tended to counterbalance FB_1-caspase-3 activity modulation by a mechanism of functional antagonism which contrasted with additive effect observed subsequently. The effect of mixture of ZEA and FB_1 on caspase-3 activity was unpredicted or in the present study, this effect is linked to the duration of cells incubations with mycotoxins.

In conclusion, our findings reveal that the combined effect of ZEA and FB_1 on cells Caco-2 seems to be unpredictable. Although, these mycotoxins could provoke the same disorders in cells, it is very difficult to predict whether their mixture can lead to antagonism, additive or synergistic effect.

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Conflict of Interest

The author(s) have not declared any conflict of interests.

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Yeast CA-11 fermentation in musts treated with brown and green propolis

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Worldwide laws are being created to reduce the amount of antimicrobial residues in foods and beverages. Distilled alcoholic beverages, such as cachaça should be detached, because some microorganisms infect the fermentation process and decrease the product quality, making it to require microbiological control. Thus, the objective of this research was to evaluate the green and brown propolis extract, as well as the previous physical-chemical treatment of sugarcane juice as antimicrobial agents and their effects on yeast CA-11 fermentation for cachaça production. The experiment was arranged in a completely randomized design in split splots with three replications. Main treatments constituted four antimicrobials (sodium monoensin, green propolis extract, brown propolis extract and physical-chemical treatment) and an untreated control. Secondary treatments were the five fermentation cycles. The amount of yeast cell and bud viability, yeast bud rate during the fermentation, pH, total acidity, glycerol and alcohol contents were evaluated in wines. The use of antimicrobial agents, especially the green and brown propolis extracts, improved the maintenance of the amount of live yeast cells and buds, and yeast budding rate as compared to the control treatment. Ethanol levels produced by the yeast strain 'CA-11' during fermentation were found to be around 6 to 7%, which are not statistically significant among the treatments. Results indicate great potential for the use of propolis as antimicrobial in fermentatiton process for production of distilled beverages, like cachaça.

Key words: Distillate beverages, cachaça, antimicrobial agents, *Saccharomyces cerevisiae*.

INTRODUCTION

To meet the market demand, technical and scientific advances are necessary, because they provide the basis for better production process control and consequently higher quality products. This is especially valid for fermented and distillated beverages to meet food quality and safety requirements in Brazil and the world (Venturini Filho, 2011).

Despite alcoholic beverages were classified by their properties (as a raw material and alcohol content), they were obtained by a biochemical process called ethanolic

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fermentation. The physical-chemical and microbiological characteristics of this process were directly related to chemical and sensorial characteristics of the beverage (Alcarde et al., 2012).

Cachaça is an important distilled beverage in the world. According to SEBRAE (2013), it is the second most consumed alcoholic beverage in Brazil and third distilled beverage in the world. Cachaça is defined as a typical and exclusive sugarcane rum beverage produced in Brazil, with alcohol content between 38 and 48% in volume; it is obtained from the distillation of sugarcane fermented must, containing unique sensorial characteristics; it has up to 6 g/L of sugars expressed in sucrose (Brasil, 2005).

Yeasts and substrates used in the fermentation process are important, because they may contain bacterial and fungal contaminants (Antonangelo et al., 2013). These microorganisms affect the fermentation biochemistry, altering must and wine compositions. Also, yeast viability is affected, resulting in lower alcohol yield and lower profitability to the industry (Nobre et al., 2007).

Many studies have been done on quality of raw material and care in processing (Cantão et al., 2010). Synthetic antimicrobials and antiseptics are frequently used during fermentation to prevent contamination. These products present different action mechanisms over one or more groups of microorganisms. However, international rules limit the use of synthetic agents because they are not compliant with food and beverage regulators with regards to residues both in the distilled beverage and yeasts (FSA, 2011). On the other hand, the use of natural antimicrobial in alcoholic fermentation processes allows residue-free production of beverages and by-products.

Propolis may therefore be an important alternative because of its wide biocide action, mainly against gram-positive and some gram-negative bacteria, through changes in bio-energetic status in cell membrane and motility inhibition. Propolis is one of the most heterogeneous and complex mixtures found in nature and contain more than 300 substances identified or characterized, as flavonoids, aromatic acids, terpenoids, phenylpropanoids and fatty acids (Arvey and Egea, 2012; Lustosa et al., 2008).

The aim of this study was to compare the antimicrobial activity of propolis extracts and physical-chemical treatment in the sugarcane juice, and their effects on fermentation performance and wine quality from the yeast strain CA-11.

MATERIALS AND METHODS

The experiment was set in Jaboticabal-SP, Brazil, during the 2013/2014 season and arranged in a completely randomized design in split-plots with three replications. Main treatments corresponded to four different methods of bacteria control [nonoensin-based synthetic antimicrobial, green propolis ethanol extract (PEE), brown propolis ethanol extract, previous physical- chemical juice treatment (PCT)]. Untreated juice was used as control. Secondary treatments corresponded to five fermentation cycles.

Sugarcane source

A third ratoon field of the sugarcane variety RB867515 was manually harvested without burning trash. Sugarcane stalks were obtained from organic certified production unit, in Jaboticabal-SP, Brazil, in October 2013.

Antimicrobial solutions preparation

The green and brown PEEs were prepared with raw propolis collected in Bebedouro- SP and Formiga-MG, Brazil (Mello et al., 2010), respectively. Determination of minimal inhibitory concentration (MIC) was performed in preliminary assays, and the concentrations used for green and brown PEE were 3 and 2 µg/mL, respectively. The PEEs were analyzed for pH (using a digital pHmeter), total flavonoids and oxidant activity (Woisky, 1996).

The synthetic antimicrobial sodium monoensin was prepared by direct dilution in 50% ethanol solution and used at a concentration of 3 µg/mL.

Must preparation

Juice was extracted by crushing cane and filtered in 60-mesh filter to remove coarse impurities (soil, cane bagasse). Juice was standardized to 16°Brix and heated to 28 - 32°C (Must 1).

To obtain must 2, the juice was clarified with adjustment of pH to 6.0 by adding calcium hydroxide (6°Bé) to obtain 16°Brix juice. The treated juice was heated until ebullition to facilitate the reaction between calcium and phosphorus found in solution (Albuquerque, 2011). The heated juice was put in inox decanter containing 5 mg/L of *Moringa oleifera* Lamarck leaf extract (Costa et al., 2014); it was prepared according to Ghasi et al. (2000), to facilitate impurity removal.

After 1 h, the supernatant was removed using a siphon. This originated the PCT, which was cooled at room temperature. The pH was not corrected during the preparation of both musts, and the natural pH of the must was considered. Juice and musts were characterized by pH, Total Reducing Sugars (TRS) (Lane and Eynon, 1934) and total acidity (CTC, 2005) analyses.

Inoculum preparation

The steps of cell multiplication, adaption and activation of flocculant yeast of *S. cerevisiae* CA-11 were conducted using a concentration of 30 g dry yeast per liter of must. Dry yeast was submitted for hydration process, using 600 mL of potable water. After 30 min, 3.0 L of sterile juice at 10°Brix was added in the fermenting vat. When the soluble solid level was 2°Brix, another 6.0 L of juice was added.

Fermentation process

Fermentation was done in batch procedure with yeast recovery by sedimentation in 6 L fermenting vat. Inoculum was prepared using 7.5% of active yeasts, previously diluted in 1.5 L of must at 6°Brix. The first vat loading was done after 30 minutes with 2.0 L, and the second after 1.5 h, with 2.5 L of must at 16°Brix. The end of the fermentation was established as 20 h after inoculation, or when the level of soluble solids was lower than 1°Brix. After the end of each fermentation cycle, 2/3 of vat volume corresponding to the wine was removed through a lateral siphon.

Table 1. Means of results obtained to total acidity in original must (1) and clarified must (2), used in fermentation cycles.

Cycle	Must 1 Total acidity (g/L H_2SO_4)	Must 2 Total acidity (g/L H_2SO_4)
1	0.73A	0.41A
2	0.61A	0.24B
3	0.58A	0.31AB
4	0.54A	0.30AB
5	0.58A	0.30AB
F test	0.97ns	4.58*
LSD	0.35	0.13
CV	21.40	15.81

*Significant at 5% of probability ($0.01 < p < 0.05$); ns = not significant; CV = coefficient of variation (%) ; LSD = less significant difference.

From the 2nd to the 5th cycle, material remaining at the bottom of the third cycle was washed with 700 mL of 0.75% sterile saline solution to remove toxic elements; and was kept without stirring. After 1 h, excess was removed and biocide treatments were applied. In the untreated control, saline solution was added again. After 1 h, new juice was fed, starting a new fermentation cycle.

In the 3rd and 5th cycle, the vat bottom was cleaned to remove inert material and dead yeast cells. Yeast cell and bud viability and budding rate were analyzed after 1 h biocides treatments, after 40 min of the second vat feeding, and at the end of the fermentation, using the method of Lee et al. (1981). Brix and pH analyses were also performed.

Wine and distilled analysis

Wines were centrifuged at 1650 x*g* and 25°C for five minutes (HIMAC CR 21G); and total acidity (CTC, 2005), pH (using a digital pHmeter) and glycerol (McGowan et al., 1993) were analyzed. Wine volatile fractions were separated through distillation (alcohol microdistiller TE-012 Tecnal); 20 mL of distillate per 60 mL of wine was recovered.

Samples were submited for alcohol content by a digital densimeter (Anton Paar DMA-48). Data were submitted for ANOVA and means were compared by Tukey test (5%), using the statistical program ASSISTAT version 7.7 beta.

RESULTS AND DISCUSSION

PEE characterization

Green and brown PEEs presented the following characteristics, respectively: pH 4.77 and 5.18 at 25°C; 0.48 and 0.70% of total flavonoids; all oxidant reaction was completed for 20 and 13 s. Results are based on the parameters established in a Brazilian technical regulation and propolis identity book ["Regulamento Técnico de Identidade e Qualidade da Própolis" (Brasil, 2001)], which show that the maxim oxidation activity is 22 s with 0.25% (m/m) of total flavonoids. Bispo Junior et al. (2012) describe that propolis antibacterial activity can be directly associated with their flavonoids content and anti-oxidant activity.

Sugarcane and must

Raw sugarcane used in the experiment had adequate technological quality for processing: 22.2°Brix, more than 90% of purity, 18.9% of total reducing sugars (TRS), 0.55% of reducing Sugars (RS), total acidity of about 0.8 g/L H_2SO_4 and pH 5.3.

After raw sugarcane analysis, extracted juice was standardized to 16° Brix; in the PCT calcium hydroxide was added and juice was heated. After dilution, must TRS decreased by 26% in relation to the extracted juice. However, these values were sufficient to activate the anaerobic metabolism in yeasts and fermentation process via Crabtree effect, which occurs when sugar concentrations in the substrate are above 6% (Venturini Filho et al., 2013).

Average pH values were found to be 5.2 in must 1, and 5.9 in must 2. This difference was expected because must 2 was treated with calcium hydroxide. The musts pH were not corrected to 4.5 before yeasts inoculation, as commonly used in production units (Cardoso, 2013), since yeast CA-11 metabolism produces by-products capable of reducing substrate pH. So, this strain does not require the sulphuric acid treatment normally used by beverage producers.

The average total acids values in musts 1 and 2 were 0.6 and 0.24 to 0.41 g/L H_2SO_4, respectively, during the five fermentation cycles (Table 1). The difference of acidity in musts is due to the previous physical-chemical treatment in juice, which removed some acids by adsorption or transport by calcium phosphates produced in solution (Albuquerque, 2011). Considering the fermentation cycles, it is observed that the PCT resulted in less reduction of these biomolecules only in the first cycle; however, the values were lower than that obtained in must 1. High concentration of these compounds negatively impacts yeast physiology, decreasing the amount of live yeast cells (Dorta et al., 2006). According to Maiorella et al. (1983), 4% of organic acids in fermentation are enough to reduce 80% of the yeast cell viability.

Fermentation process

Inoculum presented cell and bud viability higher than 90% and budding rate of 19%. These results corroborate with Lima et al. (2001) recommendations. At the beginning of the fermentation process (Figure 1A), a reduction in viable cells during the cycles was observed, mainly in the 4th and 5th cycles. However, in fermentations using musts treated with synthetic antimicrobial and brown PEE, the yeast percentage maintained a constant growth. These results corroborate with that of Oliveira Filho (2010), who tested brown PEE and press baker yeast and observed stable cell viability during five fermentation cycles, but lower viability in untreated control.

There was 8% reduction in viable yeast cells at the beginning of the 4th fermentation cycle when green PEE

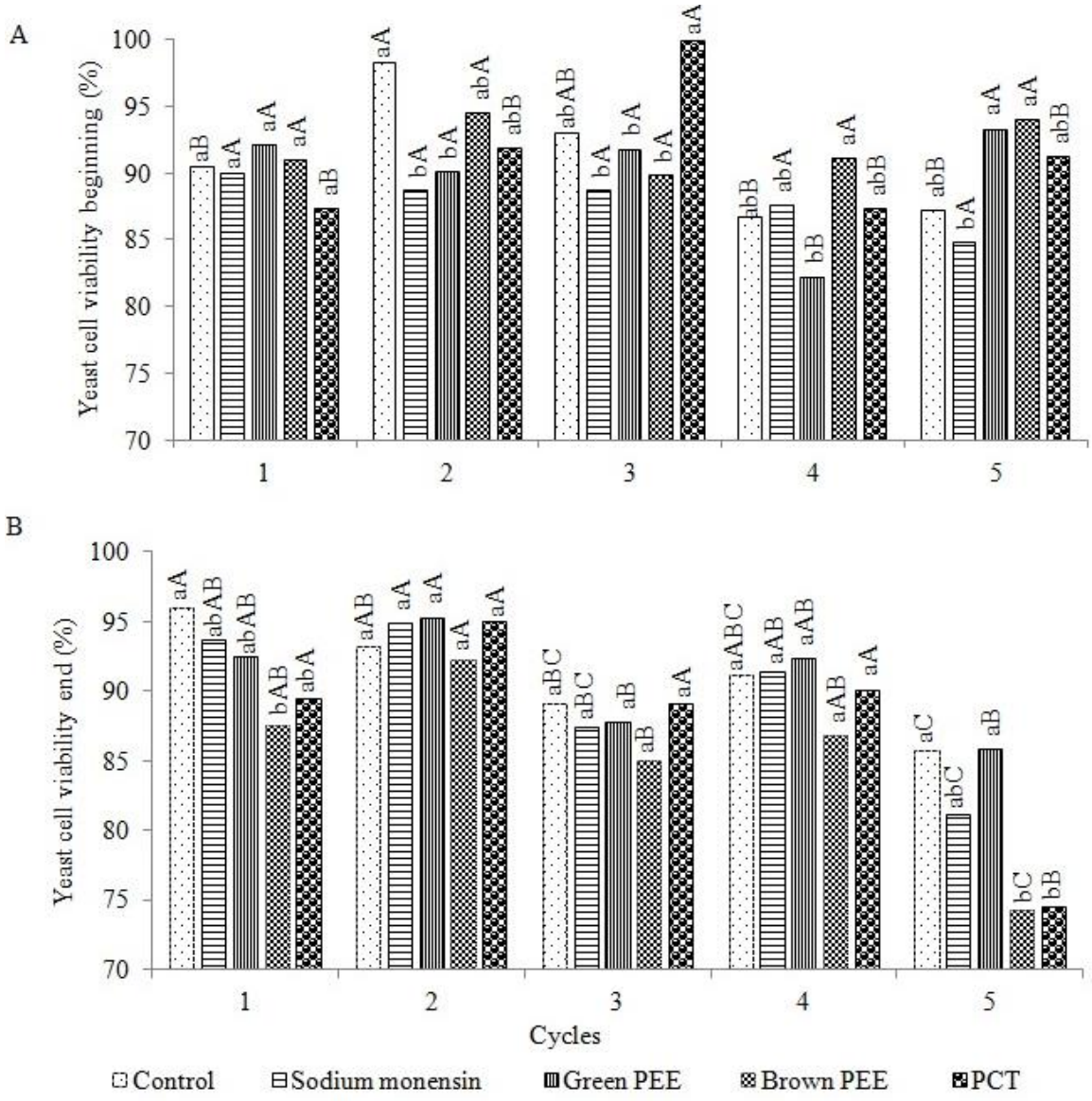

Figure 1. Interaction between must treatments and fermentation cycles for yeast CA-11 cell viability in beginning and end of fermentation process. Jaboticabal-SP-Brazil, 2013/2014season. Upper case letters compare means of the cycles. Lower case letters compare means of the must treatments in each cycle.

was used. Nevertheless, this value was about 82% and met the expectation, since an efficient fermentation requires yeast cell viability ranging from 80 to 90% (Amorim et al., 1996). In the PCT treatment, a high yeast cell viability was also observed, with values between 87 and 100%.

Also, an increase in yeast cell viability at the end of the fermentation was observed (Figure 1B) in all treatments, except in the 5th cycle, which had reductions of 84% for the check, 81% for sodium monoensin, 86% for green PEE, 74% for brown PEE and 75% for PCT. These data differ from those found by Bregagnoli et al. (2009) and Oliveira Filho (2010), who observed high cell viability only in treatments containing synthetic biocide. This divergence may occur as a result of different yeast strains or environmental conditions.

Mendes et al. (2013) related that, at the end of a fermentation cycle, the reduction in yeast cell viability is common, and frequently attributed to environmental intrinsic factors, as concentration of inhibitory metabolites such as ethanol, acids and temperature. As this was not observed in this work, it seems that the yeast strain CA-11 may have adapted to the production process environment, which favors the maintenance of its metabolic activity.

Figure 2 shows the results obtained for budding rate, at

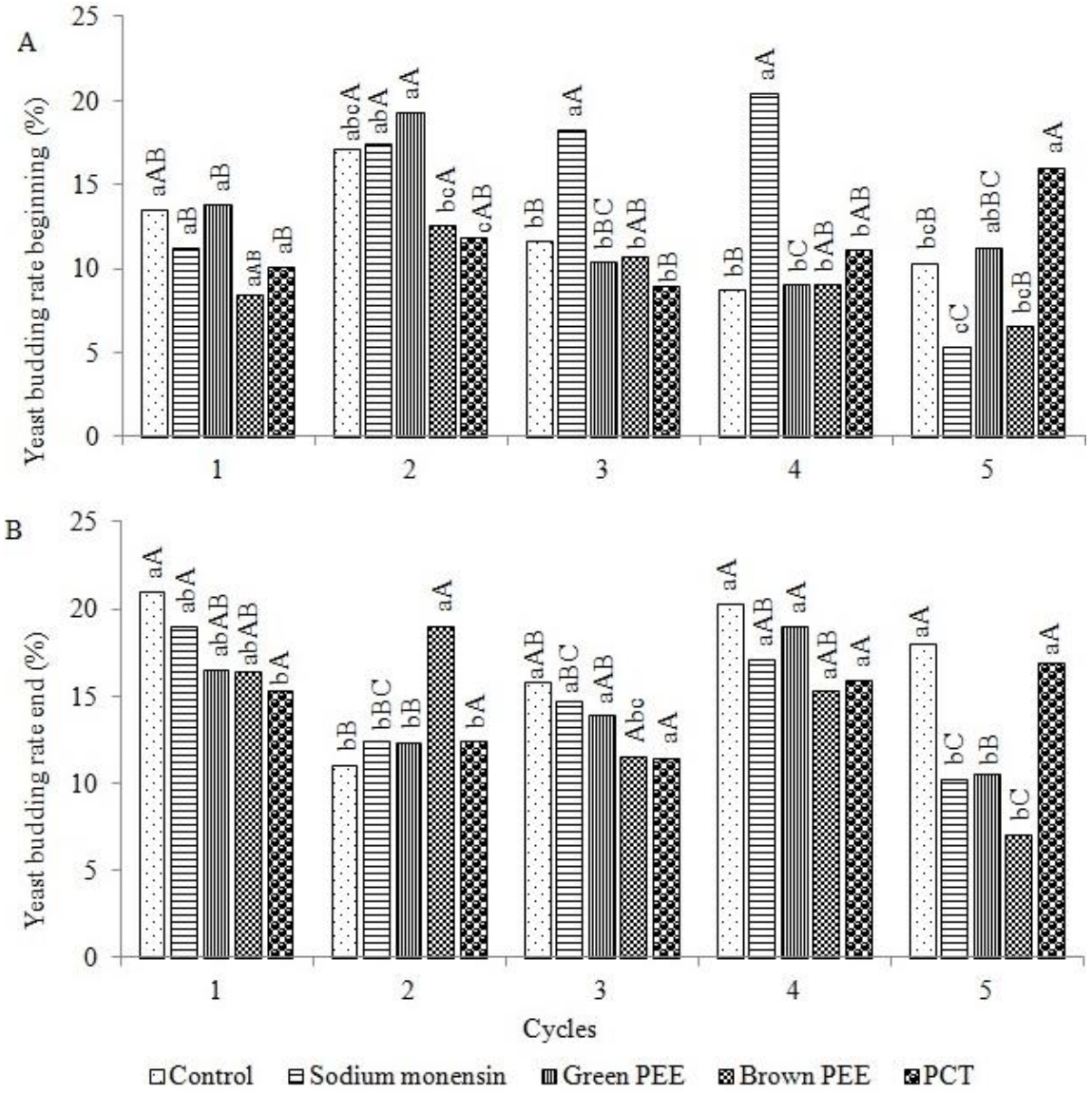

Figure 2. Interaction between must treatments and fermentation cycles for yeast CA-11 budding rate in beginning and end of process. Jaboticabal-SP-Brazil, 2013/2014 season. Upper case letters compare means of the cycles. Lower case letters compare means of the must treatments in each cycle.

the beginning (Figure 2A) and end of the fermentation (Figure 2B). During the fermentation cycles, the yeast budding rate observed ranged from 5 to 20%. These results are in accordance with Amorim et al. (1996) who verified that the adequate budding percentage in the fermentation process may vary from 5 to 15%. The use of green PEE and PCT in musts increased yeast budding rate during the fermentation cycles, but in the presence of brown PEE, the values remain stable at the end of fermentation process.

The high yeast bud rate can explain the yeast adaptability in various substrate conditions, and maintenance of cell viability was favored. However, microorganisms metabolize sugar into ethanol, glycerol or ATP and biomass production, so this high budding rate in ethanol fermentation can result in low ethanol yield and consequently the fermentation efficiency is affected (Lima et al., 2001).

Table 2 shows yeast bud viability at the beginning and end of fermentation. At the beginning of the process, the use of propolis extracts resulted in an increase of bud viability. The results of this study differ from that of Oliveira Filho (2010) and Halabi (2010), who did not find a significant difference between control, synthetic antimicrobial and propolis extract in fermentations using yeasts strains PE-2, CAT-1 and press baker yeast. In this context, a positive response of yeast CA-11 to PEE treatments was observed.

Table 2. Means of values obtained for yeast CA-11 bud viability in the fermentation cycles. Jaboticabal-SP. Season 2013/2014.

Parameter	Bud viability beginning(%)	Bud viability final (%)
Treatments (B)		
Control	88.60B	96.26A
Sodium monoensin	89.40B	89.74AB
Green PEE	93.56AB	94.62A
Brown PEE	95.97A	84.94B
PCT	91.70AB	96.55A
F test	5.80*	5.92*
LSD	5.84	9.55
CV	5.29	8.59
Cycles (C)		
1	91.86AB	91.47AB
2	88.62B	96.96A
3	92.68AB	89.78AB
4	90.07AB	95.56AB
5	96.00A	88.32B
F test	3.54*	3.67*
LSD	6.02	7.82
CV	6.28	8.11
F test (BxC)	1.67 ns	1.12ns

**Significant at 1% of proability ($p < 0.01$); *significant at 5% of probability ($0.01 < p < 0.05$); ns = no significant; CV = Coefficient of variation (%); LSD = Less significant difference.

Table 3. Means and results of ANOVA for glycerol concentration in wines.

Parameter	Glycerol (%)
Treatments (B)	
Control	0.69A
Sodium monoensin	0.59B
Green propolis	0.59AB
Brown propolis	0.48C
PCT	0.63AB
F test	12.71**
LSD	0.10
CV	14.09
Cycles (C)	
1	0.58A
2	0.57A
3	0.58A
4	0.62A
5	0,62A
F test	1.09ns
LSD	0.09
CV	15.63
F test (BxC)	1.88ns

**Significant at 1% of proability ($p < 0.01$); * significant at 5% of probability ($0.01 < p < 0.05$);, ns=no significant; CV = Coefficient of variation (%); LSD = Less significant difference.

Through fermentation cycles, there was a reduction in bud viability in the second cycle, probably associated with less cell energy production that contributes to a reduction of cell multiplication rate. However, bud viability values were higher than 88%, and considered excellent ($p < 0.05$). Yeast bud viability is important to maintain yeast population levels, because they will be reused in the next fermentation cycles, often impacting the fermentation yield (Ravaneli et al., 2006).

At the end of the fermentation, the lowest yeast bud viability was observed in brown PEE treatment. These results disagree with that of Oliveira Filho (2010), who did not observe reduction in bud viability. Although, the values are in acccordance with that of Halabi (2010), who obtained 86% of bud viability. Considering the yeast growth conditions, treatments did not show negative effects on the fermentation process.

Wine characteristics

pH and total acidity

According to Camolez and Mutton (2005), high acid levels in wine are responsible for pH reduction. This behavior was observed in treatments where the synthetic antimicrobial, green and brown PEE were applied (Figure 3A), after the second fermentation cycle. Similar results were obtained by Bregagnoli et al. (2009) and Oliveira Filho (2010), who also verified a reduction in total acidity when antimicrobial control was used.

Glycerol and Alcohol yield

Table 3 shows the glycerol concentration in wines obtained by bacterial control of the fermentation. Glycerol is synthetized by yeast to maintain the cell redox equilibrium, which is altered when organic acids and biomass production occur (Lima et al., 2001). Thus, it was observed that bacterial control resulted in less acid production and consequently the glycerol content was reduced. This performance was more evident in wines treated with brown PEE, where 0.21% of reduction was found as compared to the untreated control.

These results are higher than the observations of Ferrari (2014), who obtained results between 0.23 to 0.40% of glycerol; but lower than Balli et al. (2003), who found values of 1.10% in sucrose fermentation process at 33°C.

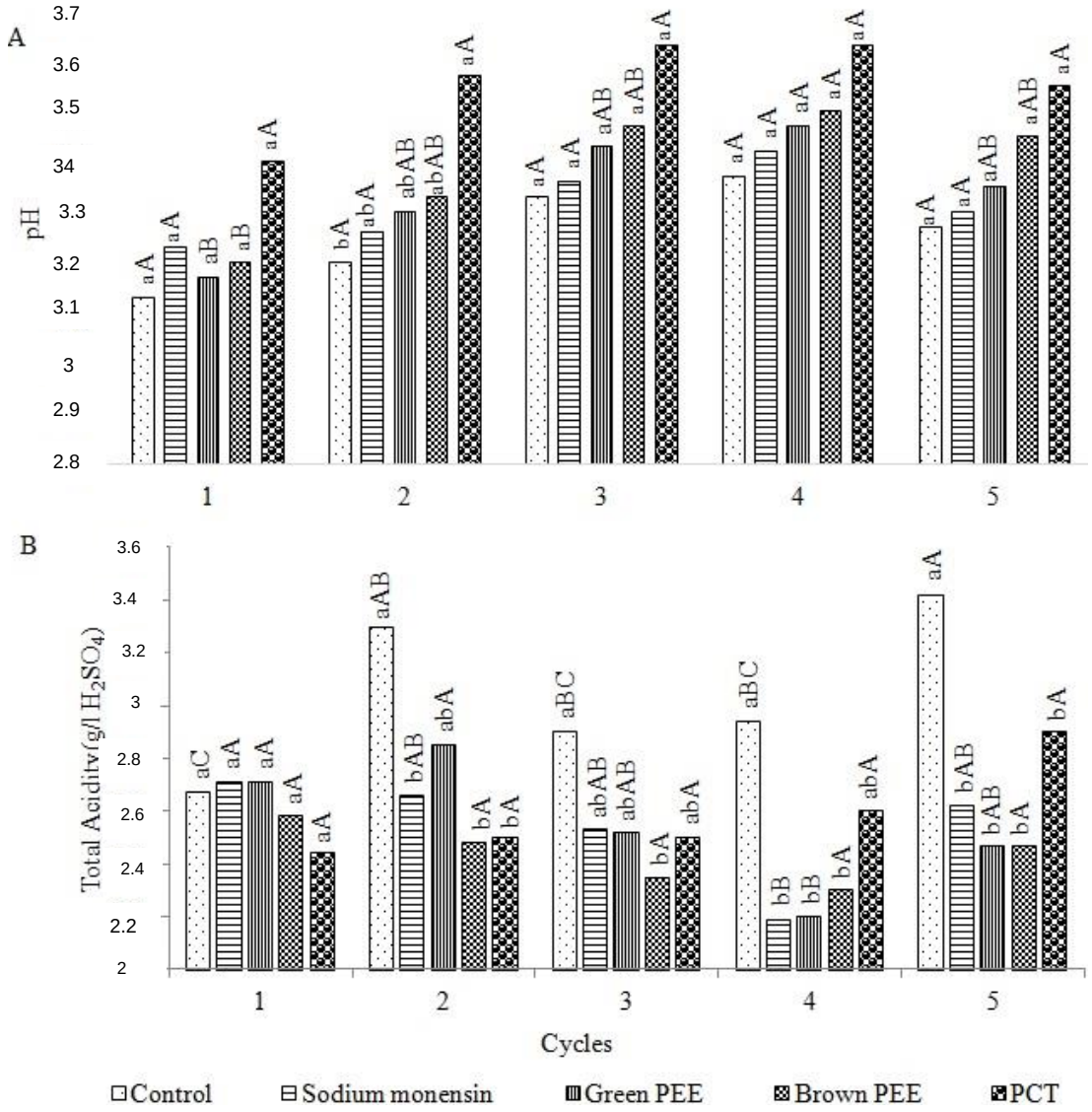

Figure 3. Interaction between must treatments and fermentation cycles for wine pH and total acidity. Jaboticabal-SP-Brazil, 2013/2014 season. Upper case letters compare means of the cycles. Lower case letters compare means of the must treatments in each cycle.

In *S. cerevisiae,* glycerol is produced in the ethanol pathway, as a by-product, competing for NADH, which is why its concentration is inversely proportional to etanol concentration. Therefore, its formation is unfavorable to fermentation process (Wang et al., 2001). Ingledew (1999) reported that up to 1% of glycerol formation is common during ethanol production process.

Ethanol rate produced by yeast CA-11 varied between 6 and 7%, and difference was not statistically significant to all treatments. Similar results were related by Bregagnoli et al. (2009) and Oliveira Filho (2010), who observed values between 6 and 7% in fermentation process using press baker yeast, and Bergamo and Uribe (2013) that used the yeast CA-11.

These results emphasize that propolis has significant antimicrobial activity to control contaminant microorganisms in alcoholic fermentations, and suggest that the use of commercial biocides can be reduced to meet the current world demand for safer food and beverage products.

Conflict of interest

The author(s) have not declared any conflict of interests.

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Full Length Research Paper

Antibacterial and antioxidant potential of polar microorganisms isolated from Antarctic lichen *Psoroma* sp.

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Recent studies suggest that bacterial communities in lichens contribute structurally and ecologically, but their biological activities are not fully investigated. In this study, we explored biological potential of microorganisms that are isolated from Antarctic lichen *Psoroma* sp. Using their bacterial cell culture extracts, we evaluated antibacterial and antioxidant properties. Among 20 bacterial species that were isolated from Antarctic lichen *Psoroma* sp., PAMC 26508 (*Streptomyces* sp., similarity: 100%) showed antibacterial activities against all target bacteria with inhibition zone diameter of 7 to 9. PAMC 26537 (*Burkholderia sordidicola*, similarity: 98.828%) exhibited higher antioxidant potential with an inhibition rate of 60.12% in 1,1-diphenyl-2-picrylhydrazyl (DPPH) and 58.69% in 2,2'-azino-bis[3-ethyl benzothiazoline-6-sulphonic acid] (ABTS) assay than control ascorbic acid (29.31%). Our results indicated that they have potential to be used as novel source of antibacterial and antioxidant agents.

Key words: Antarctica, antibacterial/antioxidant, lichen associated bacteria, lichen, *Psoroma* sp.

INTRODUCTION

Lichens are symbiotic organisms that are composed of a fungus and photosynthetic algae and/or Cyanobacteria. They can survive in extreme environments from desert to polar region because many lichens have a tolerance to extreme heat and coldness. In Antarctic ecosystems, lichens are the dominant vegetation as they are involved in soil processes, nutrient cycling and initial colonization (Lindsay, 1978). Lichens and their natural product, also called lichen substance, have been used for cosmetics, decorations, dyes, foods and medicine (Oksanen, 2006). Especially, they have been attracting attention from many researchers due to their diverse pharmaceutical potentials for antiviral, anti-proliferative, anti-inflammatory, anti-tumor and antimycobacterial activities (Lawrey, 1989; Lauterwein et al., 1995; Ingolfsdottir et al., 1998; Morita et al., 2009; Molnár and Farkas, 2010). Since they have inhibitory effect against microorganisms and high antioxidant activity, their antimicrobial and antioxidant activities are also being widely investigated (Richardson, 1998; Huneck, 1999; Muller, 2001). A few studies reported that

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lichen from polar region also have antibacterial and antioxidant activities (Paudel et al., 2008; Paudel et al., 2010; Bhattarai et al., 2013). In general, lichen substances come from the mycobiont (Stocker-Wörgötter, 2008; Luo et al., 2011).

Recent molecular studies showed that diverse bacteria are present in lichens, by the ribosomal internal transcribed spacer polymorphism and *in situ* hybridization analysis (Cardinale et al., 2006, 2008). Another research group also analyzed and compared their structure and compositions of associated bacterial communities of some lichen species, by using combined microscopic and molecular techniques (Grube et al., 2008). Despite those studies, biological activities of lichen, especially the Antarctic *Psoroma* sp. have not been studied yet, and biological activities of bacterial communities in Antarctic lichen *Psoroma* sp. are still unknown. In our previous studies, we screened 14 bacterial isolates from Arctic lichens such as *Cladonia* sp., *Umbilicaria* sp. and *Stereocaulon* sp., and antibacterial activities of these 14 bacterial isolates were determined against Gram-positive and negative clinical microorganisms (Kim et al., 2012a, 2013a). They also showed much better antioxidant activity than control ascorbic acid (Kim et al., 2012b, 2013b). The aim of this study, therefore, was to screen the antibacterial properties and antioxidant properties of 20 bacterial isolates from Antarctic lichen *Psoroma* sp. and to study this lichen as a novel source for the lichen substance.

MATERIALS AND METHODS

Collection and identification of lichen samples

The samples of lichen *Psoroma* sp. were collected in Barton Penninsula, King Geroge Island, South Shetland Island, and Antarctica (62.22 ’ S/ 58.78 ’ N) by Korean Polar Research Institute (KOPRI). They were transferred at room temperature and stored at -20°C until further use. The bacterial isolates were deposited in polar and alpine microbial collection (PAMC).

Screening of microorganisms associates with lichen

A fragment from a lichen thallus was separated by sterilized scissors or knife. Sterilized 0.85% NaCl solution was added, followed by vortexing for 10 min. After discarding the solution, the above steps were repeated. The tissue was subsequently broken with mortar in sterilized 0.85% NaCl solution. After spreading the tissue on malt extract-yeast extract (MY) agar media, international *Streptomyces* project (ISP) media, and Reasoner’s 2A (R2A) agar, it was incubated at 10°C for 15 to 21 days. To obtain pure single colony, subculturing was repeated three times, and it was preserved at -80°C in 20% glycerol. The obtained bacterial isolates were identified by their 16S rRNA gene sequences analysis. The 16S rRNA gene was amplified from a single colony of pure culture with two universal primers, 27F; 5’-AGA GTT TGA TCM TGG CTC AG-3’ and 1492R; 5’-GGT TAC CTT GTT ACG ACT T-3’, as described by Lane (1991). PCR was carried out with 25 µl reaction mixtures containing 1X PCR reaction buffer, 200 µM of dNTPs, 0.2 µM of each primer, a single colony as a template, and 1 unit of *Taq* DNA polymerase (In-Sung Science, Suwon, Korea). The PCR procedure included an initial denaturing step at 95°C for 5 min, 30 cycles of amplification (95°C for 30 s, 56°C for 30 s and 72°C for 30 s), and a final extension step at 72°C for 5 min. The PCR products were purified using the AccuPrep PCR purification Kit (Bioneer, Korea) and sequenced with the same primer used for PCR amplification. The sequence of the 16S rRNA gene was compared with that of type strains available in the database to find closely related species. All sample numbers were given by PAMC in KOPRI.

Culture and extraction of bacterial isolates

A total of five bacterial isolates were culture in 50 ml of MY, ISP and R2A liquid media at 15°C for 10 to 15 days. The culture broth was added to double volume of ethyl acetate which was analytical grade (Daejeong, Korea). Extraction was performed individually to each solvent at room temperature, and then, the layer of culture media was discarded after 2 h. The solvent layer was concentrated using rotary evaporator, and the obtained dried crude extract was dissolved in 500 µl of ethyl acetate.

Evaluation of antimicrobial activities

Test microorganisms

Ethyl acetate extracts were tested against 3 Gram-positive bacteria: *Staphylococcus aureus* (KCTC 1928), *Bacillus subtilis* (KCTC 1918) and *Micrococcus luteus* (KCTC 1915), and 3 Gram-negative bacteria: *Escherichia coli* (KCTC 2441), *Pseudomonas aeruginosa* (KCTC 1637), and *Enterobacter cloacae* (KCTC 1685). They were purchased from the Korean Collection for Type Cultures (KCTC) and Korea Research Institute of Bioscience and Biotechnology (KRIBB, Daejeon, Korea). All bacterial isolates were kept on Luria-Bertani (LB; trypton 10.0 g, yeast extract 5.0 g, NaCl 10.0 g, distilled water 1.0 L, pH 7.2) at 4°C.

Paper disk diffusion test

Paper disk diffusion test was performed according to Bauer et al. (1966) with some modifications, and all of the reagents were purchases from Difco (USA). Bacterial cells were standardized to 0.5 McFarland, and mixed with soft agar (0.04 g/ml), and 9 ml of this mixture was inoculated onto Mueller-Hinton agar plate. Then, each extract was loaded into paper disks (6 mm in diameter, ADVANTEC, Japan) and transferred onto the plates inoculated with the bacterial strains. Disks loaded with the ethyl acetate were used as a control. All inoculated culture plates were incubated at 37°C, and the inhibition zones of bacterial growth were measured after 12 to 18 h. All experiments were done twice and were compared with the control.

Evaluation of antioxidant activities

Total phenolic contents (TPC) and total flavonoid contents (TFC) test

The total phenolic content was evaluated by the Folin-Ciocalteu reagent according to the method of Slinkard and Singleton (1977) with some modifications, and all the chemical reagents were purchased from Sigma-Aldrich (USA). After the final reaction mixture was incubated for 30 min at room temperature, the absorbance was determined at 760 nm. Gallic acid was used as the positive control, and the reaction mixture without the extract was used as the negative control. The concentration of TPC was expressed in micrograms of gallic acid equivalent (GAE) per milligram

milligram of bacteria culture extract. Total flavonoid contents were evaluated by colorimetric method as described previously (Zhishen and Mengcheng, 1999). The 500 µl of lichen-bacterial culture extracts was taken, and 1.5 ml of distilled water was added to it, which was subsequently mixed with 0.15 ml of 5% $NaNO_2$ solution. After 6 min of incubation, 10% $AlCl_3$ solution was added and incubated for 6 min. Then, 2 ml of 4% NaOH solution was added to the mixture, and distilled water was added until the set final volume was reached at 5 ml. After 15 min at room temperature, the absorbance was measured at 510 nm using a spectrophotometer (Biochrome, USA). Catechin was used as a standard compound for the quantification of total flavonoids. All the values were expressed as g catechin equivalent (CE) per 100 g of extract. A single extract was measured three times.

Free radical scavenging activity using DPPH and ABTS

The free radical scavenging activity of the extract was measured by 1,1-diphenyl-2-picrylhydrazyl (DPPH) and 2,2'-azino-bis[3-ethyl benzothiazoline-6-sulphonic acid] (ABTS) reagents, and they were purchased from Sigma-Aldrich (USA). The DPPH free-radical scavenging activity of the 5 extract was determined by the method of Blois (1958) with some modifications. The reaction mixture was incubated for 30 min at room temperature. The absorbance was measured at 517 nm using a UV-Visible spectrophotometer. The reason why we used ascorbic acid instead of synthetic antioxidant (butylated hydroxyanisole, BHA; butylated hydroxytoluene, BHT; and tertiary butylhydroquinone, TBHQ) is because our extract comes from bacteria in natural lichen (Grice, 1996; Thadhani et al., 2011). The ABTS assay is also frequently used to measure antioxidant activities. The procedure followed the method of Arnao et al. (2010) with some modifications. The reaction mixture was incubated for 30 min at room temperature, after which, the absorbance was measured at 734 nm using a UV Visible spectrophotometer. Ascorbic acid of 1 mM was used as positive control, and pure solvents without the test sample were taken as a negative control in both DPPH and ABTS assay. A single extract were measured three times. Free radical scavenging activity was described as the inhibitory percentage of DPPH. ABTS was calculated according to the following equation:

$$\text{Scavenging activity (\%)} = [1 - (\text{Abs sample}/\text{Abs control})] \times 100$$

Ferric reducing antioxidant power (FRAP) assay

The FRAP assay was carried out according to the modified Benzie and Strain (1996) method and all of chemical reagents were purchased from Sigma-Aldrich (USA). The 900 µl of FRAP reagent, freshly prepared and warmed at 37°C, was mixed with 90 µl of distilled water and 10 µl of extract in different concentrations. The FRAP reagent contained 2.5 ml of a 10 mM 2,4,6-tripyridyl-striazine (TPTZ) solution in 40 mM HCl, 2.5 ml of 20 mM $FeCl_3 \cdot 6H_2O$, and 25 ml of 0.3 mM acetate buffer pH 3.6. Absorbance was measured at the 593 nm using a UV/Vis spectrophotometer. Temperature was maintained at 37°C. The readings at 30 min were selected for calculation of FRAP values.

RESULTS AND DISCUSSION

Screening for antibacterial potential of 20 lichen-associated microorganisms

A total of 20 bacteria were isolated from Antarctic lichen *Psoroma* sp., and they were identified by their 16S rRNA gene sequence analysis as shown in Table 1. It was found that the morphological and biological characteristics of the 20 bacteria were somewhat different (data not shown). Although Hidalgo group (1993) reported that pannarin, isolated from *Psoroma pallidum*, had antioxidant activity, there is no study on microbial communities and biological activity from lichen *Psoroma*. To evaluate the antibacterial potential, the paper disk diffusion test was carried out, and some of the 20 bacterial symbionts showed antibacterial activities against Gram-positive/-negative bacteria containing *S. aureus*, *B. subtilis*, *M. luteus*, *E. cloacae*, *P. aeruginosa* and *E. coli* (Table 2). The zone of inhibition diameter ranged from 7 to 11 mm, and PAMC 26508 (*Streptomyces* sp., 100% similarity) especially had antibacterial activity against all of the 6 target bacteria. PAMC 26554 (*Hymenobacter* sp., 94.661% similarity) and PAMC 26517 (*Paenibacillus* sp., 93.523% similarity) showed strongest activity against *E. coli* (11 mm) and *E. cloacae* (11 mm), respectively. All these tested strains are known as clinical isolates related to human diseases. Although there are several strong antibiotics, our results indicated that bacterial isolates from *Psoroma* sp. have the potential to be a source or a starting point for the studies on their biological compounds and treatment of related diseases.

Screening for antioxidant potential of 20 lichen-associated microorganisms

Many publications on the antioxidant activities of lichen have been reported (Behera et al., 2005; Gulluce et al., 2006; Stocker-Wörgötter, 2008; Kosanic et al., 2011; Luo et al., 2011). However, most of them used lichen or their fungal symbionts as study resource. The fact that bacterial symbionts are also present in lichen and that they contribute to lichen structurally and ecologically are previously demonstrated (Gonzalez et al., 2005; Cardinale et al., 2006, 2008; Grube et al., 2008), but biological activity of bacterial symbionts are still unexplored. Thus, we evaluated antioxidant potential of bacterial symbionts. In general, antioxidant activities are dependent on their phenolic contents and/or flavonoids contents (Gardner et al., 2000; Pietta, 2000; Halvorsen et al., 2002). Thus, we carried out TPC and TFC assay (Table 3).

TPC value ranged from 1.93 (PAMC 26557, *Rhodanobacter* sp., 97.648% similarity) to 21.01 (PAMC 26505, *Rhodanobacter* sp., 98.371% similarity) microgram of GAE per milligram of extract, and TFC value ranged from 0.73 (PAMC 26508, *Streptomyces* sp., 100% similarity) to 26.79 (PAMC 26505, *Rhodanobacter* sp., 98.371% similarity) microgram of CE per milligram of extract. Because PAMC 26505 (*Rhodanobacter* sp.) had high value of TPC and TFC among our extracts, we expected it to have the strongest antioxidant activities.

PAMC 26537 (*Burkholderia sordidicola*, 98.828% similarity) showed 60.12 and 58.69% which are the highest

Table 1. Microorganisms isolated from the Antarctic lichen *Psoroma* sp.

PAMC no.	Bacterial species (Closest strain)	16S rRNA sequence Similarity (%)	Isolation media
26505	*Rhodanobacter* sp.	98.371	R2A
26506	*Burkholderia sordidicola*	98.904	R2A
26507	*Burkholderia sordidicola*	98.921	R2A
26508	*Streptomyces* sp.	100	R2A
26509	*Burkholderia* sp.	98.534	MY
26510	*Burkholderia sordidicola*	98.928	MY
26515	*Rhodanobacter* sp.	97.771	R2A
26517	*Paenibacillus* sp.	93.523	R2A
26518	*Rhodanobacter* sp.	98.081	R2A
26519	*Rhodanobacter* sp.	98.076	R2A
26537	*Burkholderia sordidicola*	98.828	R2A
26538	*Rhodanobacter* sp.	97.648	MY
26551	*Rhodanobacter* sp.	97.771	R2A
26552	*Rhodanobacter* sp.	97.751	R2A
26554	*Hymenobacter* sp.	94.661	R2A
26555	*Frigoribacterium* sp.	97.545	R2A
26556	*Sphingomonas* sp.	97.175	R2A
26557	*Rhodanobacter* sp.	97.648	MY
26561	*Sphingomonas* sp.	97.118	R2A
26633	*Burkholderia* sp.	98.491	ISP

Table 2. Antibacterial properties of microorganisms isolated from Antarctic lichen *Psoroma* sp.

Sample no.	Gram positive			Gram negative		
	S. aureus	***B. subtilis***	***M. luteus***	***E. cloacae***	***P. aeruginosa***	***E. coli***
26505	-	-	-	-	-	-
26506	-	-	-	-	-	-
26507	-	-	7	-	-	-
26508	7	8	7	7	9	9
26509	-	-	-	-	-	-
26510	-	-	-	-	-	-
26515	-	-	7	-	-	-
26517	9	8	-	11	-	-
26518	-	-	-	7	-	-
26519	-	-	-	-	-	-
26537	-	-	-	7	-	-
26538	7	9	-	7	8	8
26551	7	8	-	-	8	9
26552	-	-	7	-	-	-
26554	9	8	-	-	-	11
26555	8	8	10	-	-	-
26556	-	-	-	-	7	-
26557	-	-	7	-	-	7
26561	-	-	-	-	7	7
26633	7	8	-	-	8	9

The property was expressed as inhibition zone diameter in mm and '-' indicates no sensitivity against target bacteria. All values are expressed as mean (n=3).

Table 3. Antioxidant properties of microorganisms isolated from Antarctic lichen *Psoroma* sp.

Sample no.	TPC*	TFC**	DPPH***	ABTS***	FRAP****
26505	25.43	26.79	26.48	18.04	2.39
26506	15.61	13.24	14.25	16.89	2.19
26507	9.84	10.02	19.64	22.31	3.27
26508	2.21	0.73	40.55	44.32	7.50
26509	16.53	10.44	17.02	19.24	3.17
26510	8.90	13.05	18.33	16.54	2.26
26515	14.26	15.04	25.00	19.27	2.35
26517	5.31	5.32	4.23	6.41	1.24
26518	10.08	11.39	23.71	24.56	4.30
26519	19.08	20.08	45.35	30.24	6.94
26537	20.21	22.36	60.12	58.69	7.59
26538	21.01	24.09	44.16	35.17	6.43
26551	19.54	14.33	25.61	22.21	4.36
26552	17.61	15.30	22.17	19.08	3.29
26554	4.77	1.59	4.97	3.85	1.31
26555	4.92	1.64	11.20	10.64	2.22
26556	2.37	6.87	50.61	55.23	8.56
26557	1.93	1.45	35.84	43.27	7.44
26561	2.04	0.98	10.98	13.28	3.07
26633	12.22	13.38	21.03	18.00	2.34

All values are expressed as mean (n=3).*TPC are expressed as gallic acid equivalents (µg GAE/mg extract); **TFC are expressed as catechin equivalents (µg CE/mg extract); ***Vitamin C uses as control (29.31%); ****FRAP are expressed as mM Fe(II)/mg extract. Ascorbic acid was used as control (6.21 mM Fe(II)/mg extract).

free-radical scavenging activity in DPPH and ABTS assay, respectively. Most of the extracts with high amount of phenolic and/or flavonoid content showed high antioxidant activities, as we have expected and as shown in the case of PAMC 26519 (*Rhodanobacter* sp., 98.076% similarity) and PAMC 26538 (*Rhodanobacter* sp., 97.648% similarity).

However, in the cases of PAMC 26508, PAMC 26556 (*Sphingomonas* sp., 97.175% similarity), and PAMC 26557 (*Rhodanobacter* sp., 97.648% similarity), their free radical scavenging activities in both DPPH and ABTS assays showed stronger activity, although they have comparably low value of TPC and TFC. It may be due to the fact that all phenolics may not have the same antioxidant activity and some of them may possess stronger activity than others. It is possible that there are synergistic or antagonistic interactions between phenolic compounds or different type of components such as carbohydrates and proteins (Rice-Evans et al., 1995).

We also carried out a FRAP assay to evaluate the reducing activity of our extract, based on the theory that antioxidant acts as reductant by performing reduction of ferric ion to ferrous ion (Benzie and Strain, 1996). So, determination of the ferrous ion formation can be used to expect the reducing power of our samples.

FRAP value of our extract varied from 1.24 to 8.56 mM in ferrous ion/mg of extract, and high amount of ferrous ion was detected in PAMC 26556 within our extract. Other extracts have lower amount of ferrous ion than ascorbic acid that was used as control (6.21 mM Fe(II)/mg extract).

Antioxidants are important in the prevention of human disease, and in generally, living organisms have a natural defense mechanism of antioxidant (Halliwell, 1997). Sometime because low level of antioxidant molecules cause damage or kill cells (Devasagayam et al., 2004), finding new antioxidant from natural sources is highly desirable. Therefore, our results revealed the possibility that the bacteria from lichen possess antioxidant activity are able to be used as new sources of natural antioxidant.

Conclusions

Antarctica is harsh environment with low temperature and nutrient restriction. Vegetation in Antarctica is limited because these conditions are not typically ideal for their growth.

Paudel et al. (2008) found that antioxidant activity of crude extract from polar lichen was more effective than other lichen species from tropical and temperate regions.

This result may relate to unique mechanisms of lichen to avoid stress from high radiation, drought and very low temperature. We expected that bacterial isolates from Antarctic lichen will follow a similar pattern because of diverse bacterial communities affecting the lichen structure and ecology. In this paper, we evaluated antibacterial and antioxidant potential of 20 bacterial symbionts isolated from Antarctic lichen *Psoroma* sp. Although we did not confirm the actual active compound, they have potential to be used as novel source of antibacterial and antioxidants. To our knowledge, this is the first study investigating the biological activity of bacterial associates isolated from the Antarctic lichen *Psoroma* sp.

Since most of identified lichen substance comes from fungal symbionts of lichen, it is a novel approach to search for new antibacterial and antioxidant compound from the nature. In addition to this, this kind of study may help to understand the unique survival mechanisms of Antarctic lichen in extreme conditions.

Conflict of interest

The author(s) have not declared any conflict of interests.

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Phenotypic characterization of *Brucella melitensis* isolated from livestock in Abu Dhabi Emirate

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Brucellosis is an infectious zoonotic disease associated with chronic debilitating infections in humans, loss of calves, lambs or kids and infertility in animals. A high prevalence of the disease in livestock is reported in Abu Dhabi Emirate. However, the isolation of *Brucella* species from livestock in this region has never been reported. Therefore, this study aimed to isolate and identify *Brucella* species involved in animal infections to the biovar level. Out of 105 samples including blood, milk and supramammary lymph nodes collected from camel, cattle, sheep and goats during the period of 2009-2010 in Abu Dhabi Emirate, a total of 30 isolates from milk samples were identified as *Brucella melitensis*. Three isolates were biotyped as *B. melitensis* biovar 1 and twenty seven isolates as *B. melitensis* biovar 3. Susceptibility testing by the disk diffusion method on Muller Hinton agar showed variable sensitivity of all strains to the 8 antibiotics tested. The study reports the first successful isolation and characterization of *B. melitensis* from livestock in the United Arab Emirate.

Key words: *Brucella melitensis*, isolation, biotyping, epidemiology, United Arab Emirate.

INTRODUCTION

Brucellosis is an infectious zoonotic disease associated with chronic debilitating infections in humans, loss of calves, lambs or kids and infertility in animals (Radostits et al., 1995). The disease is prevalent in the southern and eastern Mediterranean basin, particularly in Tunisia, Libya, Egypt, Syria, Arabian Peninsula and Iran (Pappas et al., 2006).

Globally, *Brucella melitensis* accounts for the most recorded human cases with sheep and goats emerging as an important reservoir. In contrast, few human cases due to *Brucella suis* are reported. Drinking of unpasteurized milk and milk products from cows, small ruminants or camels is considered to be the main route of human infection as well the occupational hazard (Almuneef et al., 2004). A certain percentage of the local UAE population is still drinking unpasteurised or uncooked camel and goat milk, especially in rural areas. It has been stated that in United Arab Emirates, the epidemiology of brucellosis in humans and livestock is not well understood, and available data is limited.

Bacterial load in animal muscle tissues is low but consumption of under cooked traditional delicacies such

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as liver has also been implicated in human infection (Tikare et al., 2008). Other means of human infection include skin abrasions or inhalation of airborne animal manure particles (Awad et al., 1998).

B. melitensis, one of the most virulent species of *Brucella,* is responsible for important economic losses in sheep and goats farming (Al-Majali, 2005; Montasser et al., 2011). The disease in goats is characterized by late abortion, stillbirths, decreased fertility and low milk production (Lilenbaum et al., 2007). On the other hand, sheep brucellosis can be divided into classical brucellosis, where abortion in females is the main clinical sign, and contagious epididymitis of rams caused by non-zoonotic agent *Brucella ovis* (Acha and Szyfres, 2003; Seleem et al., 2010).

Currently, despite, the high seroprevalence of animal brucellosis in Abu Dhabi emirate (Maymona et al., 2013), epidemiological information regarding biotyping of *Brucella* is unavailable, and the isolation of *Brucella* species in livestock in the emirate has never been reported. Therefore, this study aimed to isolate and identify *Brucella* species involved in animal infections to the biovar level and to determine their susceptibility to various commonly used antibiotics. This information will assist in planning for future prevention and control programs of brucellosis in Abu Dhabi Emirate.

MATERIALS AND METHODS

Collection of samples

This study was carried out during the period of 2009-2010 in Abu Dhabi Emirate, United Arab Emirates (UAE). A total of 105 samples comprised of 25 blood, 55 milk and 25 supramammary lymph nodes (L.N) were collected for bacterial cultivation from seropositive animals including 18 camels, 10 cattle, 33 sheep and 19 goats distributed across various farms located in the three regions (Abu Dhabi, Al Ain and Western region) of Abu Dhabi Emirate. Milk samples were collected from each quarter of the udder of the infected animals. Before collection, the whole udder was washed and dried; the end of each teat was swabbed with alcohol and wiped dry. A sample from each teat was milked directly into a sterile universal bottle then placed in icebox and transported to the laboratory where they were stored frozen at -20°C until cultured. Blood samples for culture were collected from the jugular veins. The collection site was cleaned with iodine solution and allowed to air-dry before 10 ml of blood were withdrawn using vaccutainer tube containing ethylenediaminetetraacetic acid, (EDTA). All blood samples were kept in an icebox and transported to the laboratory where they were immediately cultured. Supramammary L.N was collected from seropositive culled animals. Tissue samples were placed on ice in an icebox and transported to laboratory for immediate bacteriological examination or kept frozen at -20°C until cultured.

Cultivation of bacteria

Blood samples were grown in automatic BacT/Alert 3D60 systems (bioMérieux, France). An amount of 5-10 ml of each blood samples were inoculated into FAN blood culture bottles (aerobic and anaerobic) for the BacT/Alert automated sensor-metric system.

Each bottle was incubated under continuous agitation and monitored for five to seven days or until they become positive for bacterial growth. Subcultures on *Brucella* selective medium and 5% blood agar plates as well as Gram's and Modified Ziehl-Neelsen (MZN) staining were made from the positively flagged bottles

Milk samples were centrifuged at 6000-7000 *g* for 15 min at first, and then swabs from both sediments and cream were smeared onto the surface of *Brucella* selective medium (Alton et al., 1988).

The fat surrounding the L.N and adhering to the node capsules was removed from each L.N with flame-sterilized forceps and scissors. The cleaned L.N was dipped into 95% ethanol, and the ethanol was allowed to burn off. The L.N was then sliced in half exposing the inner surface and cut into small pieces with a sterile scalpel. The small pieces from the tissue of the L.N were ground in a manual tissue grinder with 1 ml of phosphate-buffered saline (PBS). The macerated tissue suspension was then streaked with a sterile swab over the entire surface of the *Brucella* selective medium.

Bacterial isolation attempts from blood, milk and L.N were made on *Brucella* Medium Base (OxoidR-CM0169) containing *Brucella* Selective Supplement (OxoidR SR83) and 5-7% horse serum. The inoculated plates were incubated at 37°C in an atmosphere of 10% CO_2 for 3-7 days for growth. Cultured plates were examined for *Brucella* organism growth on the 4^{th} day and then daily for 10 days (Alton et al., 1988).

The first subculture for milk and L.N samples together with the second subculture for blood samples were carried out using Serum Dextrose Agar medium (SDA) and Blood Agar Base medium No. 2 (OxoidR CM 271) containing 7% defibrinated sheep blood. The inoculated plates were incubated at 37°C for 24-48 h for subculture plates (Alton et al., 1988).

Characterization of bacterial isolates

Brucella colonies were examined for colony morphology and opacity by stereomicroscope (Olympus SZ61R), and acriflavine test (Sigma R A 8126) prepared at 1:1000 dilution on crystal violet for distinguishing smooth and rough colony formation (Alton et al., 1988; Corbel at el., 2005; Osterman et al., 2006). Identification of suspect *Brucella* colonies was done by the automatic identification using Vitek2 system using Gram negative (GN) cards (bioMérieux) together with the conventional biochemical tests: motility; oxidase, catalase and urease activity; glucose fermentation; Acriflavin test, staining by Crystal violet; CO_2 requirement and production of H_2S according to Alton et al. (1988).

Brucella species colonies were biotyped to the biovar level according to their agglutination pattern with monospecific A & M antisera and dye sensitivity by growth in the presence of basic fuchsin, Safranin O at final concentration of 20 µg\ml and thionin at concentration of 10 mg/ml (Alton et al., 1988; Timoney et al., 1988; Quinn et al., 2002). Bacteria phage lysis was carried out using all species of *Brucella*, brucellaphages (Tb, Wb, Fi, Iz, BK2 and R\C) at routine test dilution (RTD X 10,000) in the reference laboratory of the AHVLA- *Brucella* research, Bacteriology Department, New Haw Addlestone Surrey, UK.

Susceptibility to antibiotic test

The tests were performed according to Bauer (1966) and National Committee for Clinical Laboratory (NCCL 1990) (Quinn et al., 2002) using an antibiotic disk-diffusion assay (Hi Media, India) on Mueller-Hinton agar (Oxoid, UK). Bacterial isolates were tested against 8 antibiotics: tetracycline (30 µg), gentamicin (10 µg), chloramphenicol (30 µg), streptomycin (25 µg), vancomycin (30 µg),

Table 1. Total number of samples and *Brucella* isolates.

Sample type / animal species	Camel	Cattle	Sheep	Goat
Milk				
Total number of sample	11	10	20	14
Total number of positive samples	2	2	17	9
Percentage of positive	18.2%	20%	85%	64%
Blood				
Total number of sample	7	0	13	5
Total number of positive samples	0	0	0	0
Percentage of positive	0%	0%	0%	0%
Lymph node				
Total number of sample	7	0	10	8
Total number of positive samples	0	0	0	0
Percentage of positive	0%	0%	0%	0%

rifampicin (µg), doxycyciline (30 µg) and co-trimoxazole (25 µg). A milky suspension of each *Brucella* isolate was prepared by adding 1-2 ml of sterile phosphate buffer saline (PBS). The suspension was adjusted to McFarland turbidity standards No 5 and 1-2 ml of each suspension was used to inoculate a plate of Mueller-Hinton agar. The 8 antibiotic sensitivity discs were dispensed on each plate and then incubated at 37°C for 24-48 h in an atmosphere with 10% CO_2. The culture plates were examined after 24 h and the diameters of the zones of inhibition were measured to the nearest millimeters (mm) using a ruler across the center of the antibiotic disc. An interpretation of the size of the zone of inhibition was made according to NCCL (1990) and Quinn et al. (2002).

RESULTS

Isolation of *Brucella* species

Out of the 105 samples obtained from *Brucella* seropositive (RBT and ELISA) camels, cattle, sheep and goats, 30 *Brucella* isolates were obtained. Growth of colonies was first observed on *Brucella* selective media in 10% CO_2 as early as 3 days of incubation at 37°C and as late as 7 days and the majority of isolations were obtained by day 4. In total, *Brucella* isolates obtained were 2, 9, 17 from each of 11 camel and 10 cattle, 14 goats and 20 sheep milk samples, respectively. None of the isolates was obtained from L.N or blood samples. The rate of isolation of *Brucella* was higher among sheep (85 %), followed by goats (64%), cattle (20 %) and camel (18.2%) (Table 1).

Growth of colonies on serum dextrose agar media when examined under stereomicroscope showed smooth round colony morphology. All isolates showed typical characteristics: Gram negative short bacilli staining, non motile, non glucose fermentation, negative to acriflavin test, no retention of crystal violet, catalase, oxidase, and urease positive.

Identification and typing of *Brucella* species

The bacterial isolates were identified using the Vitek 2 compact system run in approximately 12 h with the shortest identification time being 8 h and the longest 16 h. All isolates were identified as *B. melitensis* by the Vitek 2 compact system according to the 39 built in biochemical reactions as well as one external test (oxidase test).

Culture plates of *Brucella* isolates tested for CO_2 requirement and production of H_2S provided confirmation that all the 30 *Brucella* isolates belonged to the *melitensis* species as they required CO_2, but did not produce H_2S. The thirty *B. melitensis* isolates also grew in the presence of basic fuchsin and thionin

Agglutination with both monospecific A and M antisera together with phage lysis at routine test dilution (RTD) by Tbilisi (Tb), Weybridge (Wb), Firenze (Fi) and (R\C) brucellaphages showed that, twenty seven out of the total 30 isolates were *Brucella* biovars 3, while the remaining three isolates, were recognized as *Brucella* biovar 1 (Table 2)

Antimicrobial susceptibility of *Brucella* species

All isolates showed variable susceptibility to gentamicin, tetracycline and chloramphenicol. Two isolates belonging to biovar 3 were resistant to rifampicin, doxycycline, streptomycin and vancomycin. Four isolates belongin to biovar 3 were resistant to co-trimoxazole according to the diameter of the inhibition zone (Table 3).

DISCUSSION

Diagnosis of animal brucellosis is complicated due to the

Table 2. Characterization of *Brucella* isolates to species and biovar level.

Sample\ Source	Phages at RTD					Agglutination with monospecific sera			Growth on dyes				H_2S	CO_2	Identity Species\ Biovar
	R/C	Iz	Fi	BK_2	Tb	Wb	M	A	SO	BF	TH^B	TH^A			
Milk\cow	-	-	+	+	BF	SO	+	+	Wb	NL	BK_2	NL	PL	NL	*B. melitensis* biovar-3
Milk\cow	-	-	+	+	+	-	+	+	NL	NL	PL	NL	CL	NL	*B. melitensis* biovar-3A
Milk\camel	-	-	+	+	+	+	+	+	PL	NL	CL	NL	*	NL	*B. melitensis* biovar-3A
Milk\camel	-	-	+	+	-	-	+	+	NL	NL	PL	NL	PL	NL	*B. melitensis* biovar-3
Milk\sheep	-	-	+	+	+	+	+	+	NL	NL	PL	NL	*	NL	*B. melitensis* biovar-1A
Milk\sheep	-	-	+	+	+	+	+	+	*	NL	*	NL	PL	NL	*B. melitensis* biovar-3
Milk\sheep	-	-	+	+	+	+	+	+	NL	NL	PL	NL	PL	NL	*B. melitensis* biovar-3
Milk\sheep	-	-	+	+	+	+	+	+	NL	NL	PL	NL	PL	NL	*B. melitensis* biovar-3
Milk\sheep	-	-	+	+	+	+	+	+	NL	NL	PL	NL	CL	NL	*B. melitensis* biovar-3
Milk\sheep	-	-	+	+	+	+	+	+	NL	NL	CL	NL	PL	NL	*B. melitensis* biovar-3
Milk\sheep	-	-	+	+	+	-	+	+	NL	NL	PL	NL	CL	NL	*B. melitensis* biovar-3
Milk\sheep	-	-	+	+	+	-	+	+	NL	NL	CL	NL	CL	NL	*B. melitensis* biovar-3
Milk\sheep	-	-	+	+	+	+	+	+	NL	NL	PL	NL	PL	NL	*B. melitensis* biovar-3
Milk\sheep	-	-	+	+	+	+	+	+	NL	NL	PL	NL	PL	NL	*B. melitensis* biovar-3
Milk\sheep	-	-	+	+	+	-	+	+	NL	NL	PL	NL	PL	NL	*B. melitensis* biovar-3
Milk\sheep	-	-	+	+	+	+	+	+	NL	NL	PL	NL	PL	NL	*B. melitensis* biovar-3
Milk\sheep	-	-	+	+	+	-	+	+	NL	NL	PL	NL	CL	NL	*B. melitensis* biovar-3
Milk\sheep	-	-	+	+	+	+	+	+	NL	NL	CL	NL	CL	NL	*B. melitensis* biovar-3
Milk\sheep	-	-	+	+	+	+	+	+	NL	NL	CL	NL	CL	NL	*B. melitensis* biovar-3
Milk\sheep	-	-	+	-	+	-	+	+	NL	NL	CL	NL	PL	NL	*B. melitensis* biovar-1
Milk\sheep	-	-	+	-	+	+	-	+	NL	NL	PL	NL	PL	NL	*B. melitensis* biovar-3
Milk\goat	-	-	+	+	+	+	+	+	NL	NL	PL	NL	CL	NL	*B. melitensis* biovar-3
Milk\goat	-	-	-	+	+	-	+	+	NL	NL	CL	NL	CL	NL	*B. melitensis* biovar-1
Milk\goat	-	-	+	+	+	+	-	+	NL	NL	CL	NL	CL	NL	*B. melitensis* biovar-3
Milk\goat	-	-	+	+	+	-	+	+	NL	NL	CL	NL	PL	NL	*B. melitensis* biovar-3
Milk\goat	-	-	+	+	+	+	+	+	NL	NL	PL	NL	CL	NL	*B. melitensis* biovar-3
Milk\goat	-	-	+	+	+	-	+	+	NL	NL	CL	NL	PL	NL	*B. melitensis* biovar-3
Milk\goat	-	-	+	-	+	+	+	+	NL	NL	PL	NL	CL	NL	*B. melitensis* biovar-1
Milk\goat	-	-	+	+	+	+	-	+	NL	NL	PL	NL	PL	NL	*B. melitensis* biovar-3
Milk\goat	-	-	**+**	**+**	+	+	+	+	NL	**NL**	PL	**NL**	**PL**	**NL**	*B. melitensis* biovar-3

TH^A: Thionin at 20 µg/ml (1/50,000 w/v) concentration; TH^B: Thionin at 10 µg/ml (1/100,000) concentration; BF: Basic fuchsin at 20 µg/ml (1/50,000) concentration; SO: Safranin O at 20 µg/ml (1/50,000 w/v) concentration; CL: confluent lysis; PL: partial lysis; NL: no lysis; *: some activity but not confirmed as lysis.
A: Atypical

variable incubation time and the absence of clinical signs other than abortion. Microbiological isolation of the causative organism is used to confirm the diagnosis and is considered as the gold standard method against which other tests are compared (McGiven et al., 2003). Even though cultural examinations are time-consuming and hazardous (Al-Dahouk et al., 2003; Bounaadja et al., 2009), the bacteriological isolation and identification of the etiological agent are necessary steps in the desig-nation of epidemiological and eradication programs (Refai, 2002; Zinstag et al., 2005).

Previous studies in the United Arab Emirates in general and Abu Dhabi Emirate in special revealed the wide spread of the disease among different livestock species. Moreover, all reported surveillance studies of brucellosis were based on serological tests only which may be misleading (Refai, 2002). On the other hand, to date, no attempts have been done to isolate *Brucella* species from livestock to the best of our knowledge. Therefore, the characterization of *Brucella* species currently circulating in livestock in the Abu Dhabi Emirate to their biovar level to establish the variation among them, if any, would be valuable for devising a suitable control strategy to prevent losses in the livestock.

The fact that all the thirty isolates encountered in this study from camel, cattle, sheep and goats were either biovar 1 or biovar 3 of *B. melitensis* species may be due to the type of animal husbandry practiced in the emirate

Table 3. Sensitivity of *Brucella melitensis* strains to 8 antibiotics.

Isolate no.		Antibiotic tested							
		VA30	DO30	S25	COT25	C30	R5	TE30	GEN10
B. melitensis biovar 3	M\1	T	S	S	S	S	S	S	S
B. melitensis biovar 3	M\2	M	S	S	T	S	T	S	S
B. melitensis biovar 3	M\3	R	R	T	R	T	T	T	S
B. melitensis biovar 3	M\4	T	M	R	S	S	S	M	M
B. melitensis biovar 3	M\5	M	S	S	S	S	S	S	M
B. melitensis biovar 3	M\6	M	S	S	M	M	R	S	M
B. melitensis biovar 3	M\7	M	S	T	T	S	S	S	S
B. melitensis biovar 3	M\8	R	T	R	R	T	R	T	S
B. melitensis biovar 3	M\9	T	S	S	R	S	S	S	S
B. melitensis biovar 3	M\10	M	S	S	R	S	S	S	S
B. melitensis biovar 3	M\11	S	S	S	S	S	S	S	S
B. melitensis biovar 1	M\12	T	S	T	M	S	S	S	M
B. melitensis biovar 1	M\13	M	M	S	M	M	T	S	S
B. melitensis biovar 1	M\14	S	S	T	S	S	T	S	M

GEN10 = gentamicin, TE30 = tetracycline, R5 = refampicin, C30 = chloramphenicol, COT25 = co-trimoxazole, S25 = streptomycin, DO30 = doxycycline, VA30 = vancomycin. S = Susceptible, M = moderately susceptible, T = intermediate, R = resistant.

where all animal are kept together in the same farm or due to the free animal movement between farms (Afzal and Sakkir, 1994; Moustafa et al., 1998; Maymona et al., 2013). Such result may not necessarily indicate the wide spread of this *Brucella* species throughout the United Arab Emirates rather than in one emirate of the country. Therefore, a systemic research study covering large areas and different animal species may reveal more species and biovars of *Brucella* and hence more solid epidemiological information about the disease.

In this study, the isolation of *B. melitensis* in milk is considered to be of a particular zoonotic and public health importance and suggest that, *Brucella* infection poses a threat to the public health where consumption of raw milk and milk products is commonly practiced. This is in accordance with the previous reports of isolation of *B. melitensis* from humans in Abu Dhabi emirate (Worsley et al., 1996). The unsuccessful isolation of *Brucella* from both L.N and blood in this study may be due to the chronic stage of infection in animals from which samples were collected where the organism cannot be found in blood or L.N (Richard and Kordjian, 1992).

Antimicrobial therapy of brucellosis relieves symptoms, shortens the duration of illness, and reduces complication incidence, some of which may be life-threatening. *Brucella* species have variable susceptibility to antibiotics, thus *in vitro* sensitivity should be determined (Bodur et al., 2003). The susceptibility profiled in this study were similar amongst the different isolates in their sensitivity test, which may be attributable to them all being isolated from the same geographical locality. However, resistance to a few antibiotics was noted which was probably due to individual characteristics of the organism. Generally, all isolates of *B. melitensis* biovars 1 and 3 examined were sensitivity to gentamicin, tetracycline and chloramphenicol whereas isolates of biovar 3 reacted differently in their resistance to five types of antibiotics. Antibiotic-resistant *Brucella* strains are rarely a cause of therapy failure. But, strains resistant to the main antimicrobial agents may emerge and lead to treatment failure (Cekovska et al., 2010). These differences in sensitivity and resistance of *Brucella* strains to antibiotics emphasize the significance of the use combinations of antibiotics in treatment of human brucellosis.

Conclusion

This study is believed to be the first report on the successful isolation of *B. melitensis* from livestock in the United Arab Emirate. The study demonstrates the zonootic threat of brucellosis in this region and provides a base for future programs towards preventing and controlling brucellosis in livestock and human in the area.

Conflict of interest

The authors declare that they have no conflict of interest.

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Full Length Research Paper

Antibiotic susceptibility of enteric bacteria isolated from ready-to-eat meat balangu in Kogi State, Nigeria

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Antimicrobial susceptibility of 212 enteric bacterial isolates from 60 samples of *Balangu* (ready-to-eat meat) was determined using the disk diffusion method. Based on the traditional bacteriological techniques, the enteric bacteria were identified as *Proteus* spp., *Enterobacter* spp., *Escherichia coli*, *Serratia* spp., *Klebsiella* spp., *Salmonella* spp., *Shigella* spp., *Citrobacter* spp. and *Edwardsiella* spp. Antibiotic susceptibility of these enteric bacteria showed that >90% of the isolates were resistant to nitrofurantion, cefuroxime, tetracycline and ampicillin. The resistance pattern of the isolates against nitrofurantion, cefuroxime, tetracycline and ampicillin was significant (P<0.05). Resistance of the isolates to nalixidic acid, gentamicin and chloramphenicol was as follows: 81.1, 69.8 and 62.3%. Less than 20% were resistant to ciprofloxacin and augmentin. Enteric bacteria of public health importance (*E.coli*, *Salmonella* spp., *Shigella* spp. and *Klebsiella* spp.) were observed to exhibit varying levels of antimicrobial resistance. The level of antimicrobial resistance recorded portrays an increase in the transfer of antimicrobial resistance between bacterial populations in the environment. Strict hygeinic measures should be undertaken during the processing and sale of meat and meat products to reduce microbial contamination and transmission of antimicrobial resistant bacteria to consumers.

Key words: Susceptibility, *Balangu,* enteric bacteria, antimicrobial, resistance.

INTRODUCTION

Antibiotics are extensively used in human medicine, veterinary medicine and agricultural settings in the treatment of infections, growth enhancement and prophylaxis in food animals (Barbosa and Levy, 2002). Bacterial antibiotic resistance is an emerging and serious public health concern due to the compromised efficacy of antimicrobial agents in the treatment of infectious diseases (Martinez and Baquero, 2002). Development of antibiotic resistance could be attributed to indiscriminate use of antibiotics, use of antibiotics as growth promoter in animal feed, inconsistent adherence to dosage or low dose, sub-optimal use of antimicrobials for prophylaxis and treatment of infections, etc (Byarugaba, 2009).

Aly et al. (2012) reported a significantly higher incidence of multidrug resistant *E.coli* strains from foods origin than from clinical origin. This calls for continuous monitoring of susceptibility and resistance patterns exhibited by food-borne bacteria from time to time to assist in current

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knowledge of the potency and efficacy of antimicrobial drugs for use in human and veterinary medicine.

Balangu is a processed, ready-to-eat roast beef product. In Northern Nigeria, *suya* is a very popular meat delicacy prepared and sold in a variety of forms like *Tsire*, *Balangu* and *Kilishi* (Abdullahi et al., 2005). Tsire and *Balangu* constitute the major commercially prepared ready-to-eat meat products sold on the streets of major cities and towns in Nigeria today. The consumption of this ready to eat meat product cuts across tribal and ethnic boundaries in Nigeria but predominantly prepared by the Hausas from Northern Nigeria.

Balangu is a significant source of dietary animal protein (Okonkwo et al., 1994). It is prepared by placing bulk of boneless beef on a wire mesh and roasted with fire. The roasting time is on the average about 30 min and a maximum temperature of about 70°C (Umoh, 2001).

The roasting time and temperature, moisture and exposure are probably the most important factors that have encouraged the persistence of bacteria in this meat product. Meat can serve as vehicle for disease transmission because of their nutritional composition. Studies on the microbiological quality of some of these meat products have shown that they have organisms of public health importance (Kwanga and Adesiyun, 1984; Abdullahi et al., 2005). Street-vended food industry provides employment and cheap ready to eat meals to a large proportion of the population in developing countries. It has been observed in a study that street vended foods in Mexico are potential vehicles of food borne disease because these foods are usually prepared under poor hygienic conditions of handling and selling. 40% of samples in their study were faecally contaminated and 5% harboured sufficient enterotoxigenic *Escherichia coli* which cause disease (Estrada-Garcia et al., 2002). Chao et al. (2007) conducted a study to investigate presence of *Salmonella, Listeria monocytogenes, Staphylococcus, Vibrio paraheamolyticus* and *E. coli* 0157:H7 in Chinese food products. Their data showed that raw meat, cooked products and raw milk were most commonly contaminated with food borne pathogens and many of the pathogen were resistant to different antibiotics.

This study was undertaken to evaluate the susceptibility of enteric bacteria isolated from *Balangu*, a street vended meat product to commonly used antibiotics in our setting.

MATERIALS AND METHOD

Collection of samples

Anyigba in Dekina Local Government area of Kogi State, Nigeria was our study area and the study was conducted within a period of twelve months. Sixty samples of *Balangu* were obtained from six retail locations. Temperature measurements at production points were taken with a thermometer. Moisture content was determined by placing 10 g samples of *balangu* (contained in crucibles) in an oven(50°C). Samples were weighed daily until constant weight was recorded for three consecutive days. Final weights were subtracted from initial weights. The percent difference was calculated as the moisture content (Abdullahi et al., 2005).

Bacteriological analysis

One gram of *balangu* was aseptically pounded with a sterile laboratory mortar and pestle and then homogenized in 10 ml of phosphate buffered saline for 20 min. Ten-fold dilutions were made for all samples. One milliliter of 10^{-7} dilution was plated unto sterile molten MacConkey agar (Aly et al., 2012). Inoculated plates were incubated at 37°C for 24 h. Gram staining of colonies resulting from incubation and biochemical screening of isolates (catalase, indole, methyl red, triple sugar iron and urease tests) were conducted on the isolates for preliminary identification. Bacterial isolates were subcultured on eosin methylene blue agar (EMB) and hektoen enteric agar for further identification (Bridson, 2006).

Antibiotic susceptibility

Discrete colony of 24 h old culture was taken with sterile wire loop and suspended in 10 ml of peptone water. Turbidity of the broth was made to conform to 0.5 McFarlands turbidity standard (Akond et al., 2009). The surface of freshly prepared Mueller Hinton agar (Oxoid, UK) was evenly inoculated with a drop of the culture and allowed to dry for 5 min. Gram negative multi susceptibility discs of the following antibiotics (nitrofurantoin 100 µg, agumentin 30 µg, norfloxacin 10 µg, Tetracycline 50 µg, Gentamicin 10 µg, ciprofloxacin 5 µg, chloramphenicol 10 µg, Ampicilin 25 µg, nalixidic 30 µg, cefuroxime 20 µg) was carefully placed on plates and plates were incubated for 18 h.

Zones of inhibition around each of the antibiotic were measured in millimeters. Zones of inhibition were compared with that of the National Clinical and Laboratory Standard institute breakpoints for each of the antibiotics used (CLSI, 2007). *E. coli* ATCC25922 was used as control.

Statistical analysis

The Chi-square Test (χ^2) was used to determine the level of significance of the different antibiotics to the microorganisms isolated from *'Balangu*'. The level of significance was set at $p<0.05$. For the purpose of statistical analysis, the intermediate and sensitive isolates were grouped together as being susceptible.

RESULTS

Enteric bacteria isolated from *Balangu* are listed in Table 1 according to the frequency of isolation. Most commonly occurring enteric bacteria were *Enterobacter* spp. and *E. coli* (19.8 and 16.0%, respectively), the least occurring enteric bacteria was *Edwardsiella* spp. (1.9%). *Salmonella* spp. and *Shigella* spp were isolated at 4.7 and 2.8% frequency, respectively.

Susceptibility pattern of '*Balangu'* isolates to ten antibiotics is shown in Table 2. More than 98% of the isolates were resistant to nitrofurantion and cefuroxime. Resistance to tetracycline and ampicillin was 96 and 94%, respectively. There was a significant difference ($p < 0.05$) in the resistance of the enteric bacteria isolated to nitrofurantion, cefuroxime, tetracycline and ampicillin.

Antimicrobial resistance to nalixidic acid, gentamicin and chloramphenicol were 81.1, 69.8 and 62.3%. Seventeen percent of the isolates were resistant to ciprofloxacin and

Table 1. Frequency of isolation of enteric bacteria in *Balangu* (n=60).

Bacteria	Frequency	%
Proteus vulgaris	24	40.0
Proteus mirabilis	14	23.3
Enterobacter spp.	42	70.0
Citrobacter spp.	10	16.7
E.coli	34	56.7
Serratia spp.	10	16.7
Klebsiella spp.	20	33.3
Shigella spp.	6	10.0
Salmonella spp.	10	16.7
Flavobacterium spp.	8	13.3
Edwardsiella spp.	4	6.7
**Pseudomonas* spp.	30	50.0

*Not an enteric bacteria but commonly isolated.

Table 2. Antimicrobial susceptibility of enteric bacteria isolated from *Balangu* (n= 212).

Antibiotic	Resistant (%)	Intermediate (%)	Sensitive (%)
Nitrofurantoin	208 (98.1)	4 (1.9)	0 (0)
Augumentin	36 (17.0)	144 (68.0)	32(15.0)
Norfloxacin	104 (49.1)	108 (50.9)	0 (0)
Tetracycline	204 (96.2)	4 (1.9)(1.9)	4
Gentamicin	148 (69.8)	4 (1.9)	30(14.2)
Ciprofloxacin	36 (17.0)	120 (56.6)	56(26.4)
Chloramphenicol	132 (62.3)	80 (37.7)	0 (0)
Cefuroxime	208 (98.1)	4 (1.9)	0(0)
Nalixidic acid	172 (81.1)	40 (18.9)	0(0)
Ampicillin	200 (94.3)	12 (5.76)	0 (0)

augumentin. Antimicrobial susceptibility of 4 enteric bacteria of public health significance namely *E. coli, Shigella* spp., *Salmonella* spp. and *Klebsiella* spp. was also evaluated. Results showed that these bacteria were more susceptible to ciprofloxacin, augumentin and norfloxacin. Susceptibility of these bacteria to the other antimicrobial agents was however observed to be low (Table 3).

DISCUSSION

The results of antimicrobial susceptibility of enteric bacteria isolated from balangu reveals a high level of resistance to seven of the antimicrobial agents which are commonly used in the treatment of infections caused by Gram negative bacteria. This portrays a possible transmission of antimicrobial resistant (AMR) bacteria to balangu. The raw beef used in the preparation of this product, environment and handlers are possible sources of these AMR bacteria. AMR bacteria have been found in various food products, environmental samples and even in hosts without a history of direct exposure to antibiotics (Wang and Schaffner, 2011). The existence of large antibiotic resistance gene pools in foodborne commensal bacteria present in many ready-to-consume food items suggests that human beings are constantly being inoculated with large numbers of antibiotic resistant bacteria through daily food intake independent of clinical antibiotic exposure (Duran and Marshall, 2005).

Extremely high AMR to nitrofuratoin and cefuroxime may have been as a result of the lower concentration of the two antimicrobial agents. CLSI break point was 300 µg for nitrofuratoin and 30 µg for cefuroxime (CLSI, 2007). The more pronounced AMR observed for tetracycline and ampicillin is consistent with the report of Kariuki et al. (2006) on a significant proportion of non-typhoidal *Salmonella* being multiple and resistant to ≥ 3 antibiotics including ampicillin and tetracycline. Tadasse et al. (2012) found co-resistance for tetracycline with ampicillin and chloramphenicol in their study of antimicrobial

Table 3. Frequency of antimicrobial resistance of some enteric bacteria of public health importance isolated from *Balangu* (%).

Antimicrobial spp. agent	*E. coli*	*Salmonella*	*Shigella* spp.	*Klebsiella* spp.
Nitofurantoin	94.1	100	100	95.0
Augmentin	0.0	10.0	16.7	10.0
Norfloxacin	17.6	20.0	100	15.0
Tetracycline	67.6	40.0	100	60.0
Gentamicin	44.1	20.0	66.7	15.0
Ciprofloxacin	2.9	10.0	0.0	10.0
Chloramphenicol	91.2	40.0	83.3	5.0
Cefuroxime	97.1	90.0	83.3	90.0
Nalixidic acid	41.2	50.0	100	30.0
Ampicillin	88.2	90.0	100	80.0

resistance of *E. coli* from humans and food animals. The use of antibiotics in treatment and as growth promoters in food animals has been implicated in the increasing number of antibiotic resistant enteric bacteria in meat and meat products. The low AMR recorded for augmentin and ciprofloxacin suggests that these antimicrobial agents may still exert some antimicrobial properties against intestinal bacterial pathogens. Studies conducted by Tadasse et al. (2012), Karczmarczyk et al. (2011) and Zinnah et al. (2007) have also reported low AMR to augmentin and ciprofloxacin.

In conclusion, a continuous monitoring of antimicrobial susceptibility of bacteria connected with meat and meat products is beneficial in formulating control strategies for antimicrobial resistance which has become a worldwide concern due to its ever increasing status.

Conflict of Interest

The author(s) have not declared any conflict of interests.

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