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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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African Journal of Biotechnology

Table of Contents: Volume 14 Number 41, 14 October, 2015

ARTICLES

Heterogeneous expression and functional evaluation of in *silico* characterized recombinant OmpC of *Salmonella* Typhimurium as a functional poultry vaccine to eradicate zoonotic transmission 2862
Richa Jha, Anil Kumar, Anjani Saxena, Mamta Pandey, Rajesh Kumar and Mumtesh Kumar Saxena

Use of simple sequence repeat (SSR) markers for screening blue disease resistance in cotton germplasm exchange 2871
Christopher Faustine, Lucia Vieira Hoffmann, Flora Ismail Tibazarwa and Everina Lukonge

Antibiosis in *Ascia monuste orseis* Godart (Lepidoptera: Pieridae) caused by kale genotypes 2876
Luciano Nogueira, Lígia Alves de Paiva, Andre Cirillo de Sousa Almeida, Zulene Antonio Ribeiro Arlindo Leal Boica Junior and Flavio Goncalves de Jesus

Estimation of genetic diversity between three Saudi sheep breeds using DNA markers 2883
Ahmed Abdel Gadir Adam, Nada Babiker Hamza, Manal Abdel Wahid Salim and Khaleil Salah Khalil

Determination of lactic acid bacteria in Kaşar cheese and identification by Fourier transform infrared (FTIR) spectroscopy 2890
İlkay Turhan and Zübeyde Öner

Profile, antioxidant potential, and applicability of phenolic compounds extracted from *Spirulina platensis* 2903
Taiana Denardi de Souza, Luciana Prietto, Michele Moraes de Souza and Eliana Badiale Furlon

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African Journal of Biotechnology

Full Length Research Paper

Heterogeneous expression and functional evaluation of *in silico* characterized recombinant OmpC of *Salmonella* Typhimurium as a functional poultry vaccine to eradicate zoonotic transmission

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Salmonellosis is one of the major global health concerns leading to millions of deaths annually. The present vaccines not being up to the mark necessitate the need for the development of new generation vaccines. Outer membrane proteins (Omps) of several Gram negative bacteria have been investigated and found to be immunogenic and protective. The present study explores the potential of a major porin protein (OmpC) of *Salmonella* Typhimurium, as a vaccine candidate. The OmpC 3D structure and its potential to bind effectively with antibodies and generate humoral response was investigated using *in silico* docking, and expressed in a heterogeneous *Escherichia coli* M15 host strain. The rOmpC was purified and its immunopotential was evaluated *in vitro* by western blotting and *in vivo* in three weeks old chicks. The recombinant OmpC produced a significant humoral response and in vaccinated birds 100% survival rate was observed along with delay in the shedding of organism in droppings. These findings indicate that the rOmpC vaccination prevents mortality in chicken and lowers fecal shedding in droppings.

Key words: Outer membrane protein (Omp), Salmonellosis, *Salmonella* Typhimurium, subunit vaccines, porins, rigid-body docking.

INTRODUCTION

Salmonellosis is a major health concern affecting a large population globally. *Salmonella* enterica, the causative agent of Salmonellosis results in food borne illnesses and gastroenteritis in human beings and other mammals (Sharma, 2003). Due to its high physiological adaptability and broad host range it is difficult to devise prevention and control strategies against Salmonellosis. Although, the incidence rate of typhoidal Salmonellosis has been significantly controlled by vaccination and drugs, but non-typhoidal species continue to cause infections at a steady

rate in the last 15 years as indicated by Department of Health (2011). The major serovars contributing to the prevalence of Salmonellosis worldwide are *Salmonella* Typhimurium and *Salmonella* Enteritidis (Ekdahl et al., 2005; Laupland et al., 2010). Globally, non-typhoidal Salmonellosis results in over 1.3 billion cases and 3 million deaths annually (Pui et al., 2011), poultry products being the major source. The limitations in curbing the zoonotic transmission of such non-typhoidal serovars are a major drawback in the field which poses a risk to animal as well as human health. Therefore, it is essential to develop effective vaccination and strict biosecurity measures in such a manner that the transmission of *S. Typhimurium* through poultry is halted to break the circle of continuous infections. Hence, in this study recombinant antigenic product from *S.* Typhimurium is studied as a poultry vaccine which can strongly inhibit the zoonotic transmission.

Salmonella serovars were used to produce killed bacterins (Nicholas and Andrews, 1991) and attenuated vaccines by culturing the vaccine strain in iron-deficient medium (Woodward et al., 2002). Killed vaccines are usually less effective as they only contain surface antigens that give an incomplete protective antibody response, they fail to elicit cell-mediated immune response and they fail to elicit production of secretory immunoglobulin (sIgA) response. Attenuated vaccines have drawbacks like immune-suppression and fecal shedding in vaccinated animals (Barrow and Wallis, 2000). Moreover, the recent emergence of antibiotic resistance and reversal of pathogenicity in *Salmonella* further strengthen the requirement of an effective vaccine management of Salmonellosis. Several bacterial components like adhesive proteins, polysaccharides, lipoproteins and outer membrane proteins have been investigated as immunogens (Yap et al., 2001; Lin et al., 2001; Wolfenden et al., 2010; Bouzoubaa et al., 1987, 1989) which may be protective. Such factors which interface the cell with the environment may be efficiently used as immunogens so as to stimulate the development of protective immunity. The outer membrane proteins (Omp(s)) of *Salmonella* are considered functional candidates as they represent crucial virulence factors with a significant role in the pathobiology of gram negative bacteria and bacterial adaptation (Hamid and Jain, 2008).

Porins represent the most abundant class of Omp(s) that have been elucidated to be protective (Calderon et al., 1986; Isibasi et al., 1988) in few serovars of *Salmonella* (Prejit et al., 2013). They have important role in the maintenance of bacterial physiology aiding transport of small hydrophilic molecules (Nikaido, 1996), having a potential role as immunogens in diagnostic assays and vaccination (Hamid and Jain, 2008; Aron et al., 1993; Secundino et al., 2006; Singh and Sharma, 1999). Specific humoral and cellular immune responses are mounted against *Salmonella* Omp(s) (Aron et al., 1993; Secundino et al., 2006; Begum et al., 2008; Bhat and Jain, 2010; Prejit et al., 2013). Several outer membrane *Salmonella* proteins have been characterized, evaluated and found to be immunodominant in poultry (Wyszynska et al., 2004; Begum et al., 2008; Meenakshi et al., 1999; Nicholas and Andrews, 1991).

Outer membrane proteins when administered together were found to control fecal shedding of organisms in vaccinated birds (Meenakshi et al., 1999; Prejit et al., 2013). These porins of *Salmonella* contain T cell immunodominant antigenic regions and are involved in providing protection in typhoid fever and murine salmonellosis (Quinonez et al., 2004). OmpC, a major porin protein was found to be synthesized and incorporated into the outer membrane at both low and high osmolarity conditions (Arockiasamy and Krishnaswamy, 2000) depicting its role in both free living condition and infection. It is a good candidate to display heterologous epitopes on the cell surface (Puente et al., 1995; Arockiasamy et al., 2004a) and the detailed structural properties have been clearly elucidated B (aalaji et al., 2006; Baalaji et. al., 2006). The immunogenic potential was earlier studied and it was observed to be imunodominant and thermostable protein (Verma et al., 2009; Jailkhani et al., 2007). Hence, OmpC is considered as a potential candidate for the development of a subunit vaccine against Salmonellosis (Prejit et al., 2013). Further, OmpC-OmpF mutant was found to possess attenuated virulence (Chatfield et al., 1991) revealing its role in pathogenesis and was also observed to confer long lasting and specific humoral response against the pathogen (Secundino et al., 2006).

As the isolation of Omp(s) is a tedious task, production of recombinant proteins has been considered as an alternative improved strategy. The present study deals with the evaluation of OmpC as a vaccine candidate. Initially, 3D structure, epitope mapping and docking studies were conducted so as to evaluate the immune-potential of the protein *in silico*. The protein was further expressed in a heterogenous system, purified and analyzed by SDS-PAGE and Western Blotting for *in vivo* evaluation in model organisms. The *in vivo* studies of recombinant protein in chicken, further illustrates its role as a vaccine candidate with a need for a more detailed study.

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Abbreviations: Omp C, Outer membrane protein C; **PVDF,** polyvinylidine fluoride; **OPD,** orthophenyl diamine; **TT,** tetrathionate.

MATERIALS AND METHODS

Bacterial strains

The culture of *S. enterica* subsp. *enterica* serovar Typhimurium MTCC 3231 was procured from Institute of Microbial Technology (IMTECH, Chandigarh). The *Escherichia coli* M15 cells were used for the expression of recombinant protein.

Animals

Twenty one days old White Leghorn chicks were procured from Instructional Poultry Farm, G.B. Pant University of Agriculture and Technology, Pantnagar. All experiment were approved and in accordance with the guidelines of Committee for the purpose of Control and Supervision of Experiments on Animals (CPCSEA). The birds were reared under hygienic condition and provided with feed and water *ad libitum*.

Verification of *ompC* clone

The *ompC* gene from *S.* Typhimurium MTCC 3231, was cloned in pJET1.2 blunt cloning vector using primers: Forward primer—5' GGATCCATGCGTATCGGCTT 3', Reverse primer—5' AAGCTTTTAGAACTGGTAAA 3' (Clone JET^{TM} PCR cloning kit, Fermentas, USA) (Jha et al., 2012) and sequenced by Ocimum Biosolutions Ltd., Hyderabad. The recombinant plasmid was purified by alkali lysis method (Sambrook and Russell, 2001), and was verified by double digestion using *Bam*HI and *Hind*III.

Expression of recombinant 38.5 kDa OmpC protein

The 1.0 kb gene for 38.5 kDa OmpC was directionally subcloned in pQE30 expression vector (Khanam et al., 2006) with the 6X His Tag. The expression construct was transformed in *E. coli* M15 host cells. Recombinant strains were maintained in Ampicillin (50 µg/ml) and Kanamycin (25 µg/ml) and induced with 1 mM isopropylthiogalactoside (IPTG) after OD_{600} reached 0.5 to 0.7 (Sambrook and Russell, 2001). The expression of recombinant protein was analyzed on SDS-PAGE and the molecular weight was determined.

Purification of recombinant 38.5 kDa protein by Ni-NTA affinity chromatography

The cell lysate was loaded onto the column of His-Select Nickel Affinity gel (Ni-NTA resin, Sigma, USA), pre-equilibrated with equilibration buffer (100 mM NaH_2PO_4, 10 mM Tris base, 8 M Urea, pH 8.0). The column was washed with 25 column volumes of wash buffer (pH 7.5), the bound fractions were eluted with 5 ml of elution buffer (pH 4.5) and analyzed by 12.5% SDS-PAGE and quantified using Lowry's method (Lowry et al., 1951).

In silico analysis of *ompC* gene sequence

The *ompC* gene sequence (Acc. No. JF896322) was characterized and the putative 3-D structure was determined. Structural analysis was conducted using EsyPred3D (Lambert et al., 2002) for determination of the 3-D structure. Jmol was used as a visualization tool. The Ramachandran plot of the sequence was predicted and the plot statistics was analyzed as suggested by Morris et al. (1992).

Rigid body docking prediction of OmpC recombinant protein with representative antibodies

The receptor antibody models were extracted from Protein Data Bank to study the potential ability of OmpC to strongly bind with the antibodies. HexServer (Macindoe et al., 2010) was used for rigid body Antigen-Antibody docking using OmpC 3D structure, with Range angles of 180° and Step sizes at 7.5. A non-specific 6D docking run was conducted using default values for all parameters. Since the macromolecules have shape and charge complementarity, shape plus electrostatic calculations were made. The server screens the possible complementarities and clusters the solutions with similar orientations for shape plus electrostatic correlations (N = 25 or N = 30) to give sharper results. The best docking predictions were visualized and the docking energy was determined to evaluate the stability of the *in silico* complexes.

Raising hyperimmune sera against total Omp(s)

The Omp(s) of *S.* Typhimurium were isolated as per the protocol described by Choi-Kim et al. (1991). Briefly, 1 L of *S.* Typhimurium culture suspension was sonicated in 10 mM HEPES buffer (pH 7.4). The suspension was centrifuged at 1,00,000 xg for 60 min at 4°C and washed in 2 ml of 2% (w/v) sodium lauryl sarcosinate. Hyperimmune serum was raised in New Zealand White rabbit. The animal was subcutaneously injected with 500 µg of Omp(s) emulsified in Freund's complete adjuvant, followed by 250 µg of protein as first and second booster at 10 and 21 days, respectively. Serum samples were collected from ear vein at seven days post final booster. Agglutination reaction was performed on a slide with a layer of 1% agarose in normal saline. Three wells were cut using a well borer at a distance of 1 cm from each other. 50 µl of total Omp(s) was added to one well, 50 µl of serum was added to the middle well and 50 µl autoclaved water was added to the last well. The slide was kept in a moist chamber for 48 h after which agglutination was observed.

Western blotting

Purified recombinant protein was electroblotted on polyvinylidine fluoride (PVDF) membrane using semi-dry method at 0.8 A/cm^2 as described by Towbin et al. (1979). The membrane was blocked with 1% BSA, incubated with Omp antiserum at 37°C for 1 h, washed and incubated again with anti-rabbit HRP conjugated antibodies at 37°C for 1 h. Further, substrate solution (50 mM Tris-HCl pH 7.6, 0.6 mg/ml 3-3' Diaminobenzidine, 0.6 µl/ml 30% H_2O_2) was added and the signal was developed and analyzed.

Immunization and humoral response in poultry

Thirty (30) white Leghorn birds, 21 days old were divided into two equal groups for immunization studies. Group I was subcutaneously injected with 100 µg saponin from quillaja bark (Sigma Aldrich) in phosphate buffer saline as control Group II was injected with 150 µg of recombinant purified protein formulated with 100 µg saponin per bird. Two injections at 0th and 21st day were followed by a booster dose on 28th day and the blood samples were collected from wing vein on 7 and 14th day post immunization. The serum samples were analyzed for antibody titre using ELISA. The OmpC protein was coated (100ng/well) into the wells of ELISA plate and kept at 4°C for overnight. After washing, 100 µl of blocking solution (2% BSA in PBS) was added and the plate was kept at 37°C for 1 h. The plates were washed thrice with washing solution (PBS containing 0.05% Tween-20). Hundred µl of serially diluted serum

was added and the plates were incubated for two hours at 37°C. After third washing 100 µl of anti-mouse HRP conjugated antibody diluted 1:3000 times was added and the plates were kept in incubation for one hour at 37°C. The substrate solution (24.3 ml of 0.1 M citric acid, 25.7 ml of 0.2 M dibasic sodium phosphate solution, 40 mg orthophenyl diamine (OPD), 0.04 ml of hydrogen peroxide and 50 ml of triple glass distilled water) was prepared during the time of incubation. The plates were washed thrice with washing solution. Then, 100 µl of OPD substrate was added and the plates were in incubated at room temperature in dark for 30 min. Then 100 µl of 1.5 N NaOH was added to each well. The OD was observed at 492 nm. Two weeks post immunization; birds were challenged with 100 µl culture containing 10^9 cfu of *S.* Typhimurium 3231 culture strain intraperitoneally. The challenge strain administered intraperitoneally allows the development of clear symptoms, lethargic behavior and mortality. The birds were monitored for clinical signs, mortality and shedding of challenge strain using cloacal swab cultures for 10 days. The cloacal swabs were collected on 5^{th} and 10^{th} day post challenge, enriched in tetrathionate (TT) broth and verified on Brilliant Green Agar to estimate shedding of pathogen strain form host system.

Statistical analysis

The statistical analysis for comparison between mortality and shedding rates was performed using paired and unpaired Student's t-test and the mean titre was calculated by deriving the mean of logarithmic value of reciprocal of titre with standard error. The results of challenge studies were analyzed using Chi square test with Yate's correction.

RESULTS AND DISCUSSION

Heterologous expression of *ompC* gene

The expression cassette was verified by restriction analysis and transformed in heterologous *E. coli* M15 cells. The recombinants were screened for the expression of recombinant OmpC by analyzing mini prep cultures on SDS-PAGE.

The recombinant protein (rOmpC) was found to express effectively under the regulated control of IPTG as inducer. The induced protein appeared to be of 38.5 kDa and the expression was found to be significantly high indicating a large proportion of the protein synthesis machinery being directed for the expression of recombinant protein.

Purification and quantification of rOmpC

The rOmpC was purified using Ni-NTA affinity chromatography and recombinant protein eluted at pH 4.5 was found to be almost pure in SDS-PAGE analysis (Figure 1b). The protein yield of 30 g/L (3 g/100 ml) of bacterial culture was obtained which was estimated by Lowry's method and further concentrated by dialysis. The high expression level of the rOmpC would aid in generating immunodiagnostics and vaccines.

3D structure predictions of rOmpC protein and rigid body molecular docking with representative antibodies

The protein was predicted to have 15 antiparallal β-sheets forming a β- barrel characteristic of Gram negative bacterial porins. Five alpha helical regions were observed distributed in the protein sequence. Out of them three were found to be located on the extracellular surface of the membrane. One was localized in the hydrophillic loop region directed intracellularly. The fifth helix was found to be located within the pore of the protein. It probably contributes in providing a hydrophilic surface within the pore, for the effective transport of hydrophilic molecules. The β- barrel region of the protein constitutes the transmembrane region being hydrophobic in nature. *In silico* studies revealed pore size of ~1.25 nm (Figure 2a, b) which was slightly more than 1.1 nm as predicted by Baalaji et al. (2006), for *S.* Typhi (Morris et al., 1992). The Ramachandran plot showed the phi-psi torsion angles for all residues in the structure (except those at the chain termini) (Figure 2c). The darkest areas "core" regions (red) represented the most favourable combinations of phi-psi values (Morris et al., 1992). It was observed that 86.2% of the residues were present in the favoured regions except Tyrosine -90 and aspartic acid -273 in the fourth quadrant, although no residues were found in the disallowed regions of the plot (Supplementary 1) indicating stable and favourable protein structure. Further, it was observed that the regions in the sequence having high surface probability, have a high antigenic index and the transmembrane regions were found to be highly conserved in *Salmonella* and *E. coli* (Puente et al., 1995).

Interaction of OmpC antigen with antibody receptors of surface proteins

To study the antigenic potential of the protein *in silico*, eight antibody models against surface proteins were selected and extracted from PDB. The Docking was done using HexServer (Macindoe et al., 2010) (Table 1) and binding energies were obtained. These binding energies correlated with high stability and shape complementarities between the receptor and ligand 3D structures (Figure 3a-h). The negative values predicted the stability and strength of rigid body docking predictions. Since, the binding energy is indicative of interaction energy, Vander Waals forces and hydrophobic interactions, the conclusion was made that the antigen may be a probable ligand for such antibodies within the host system. The *in silico* energy correlations emphasize the predictive immunogenic property of the recombinant antigen and its probable interaction with B-cell receptor molecules generated during adaptive immune responses.

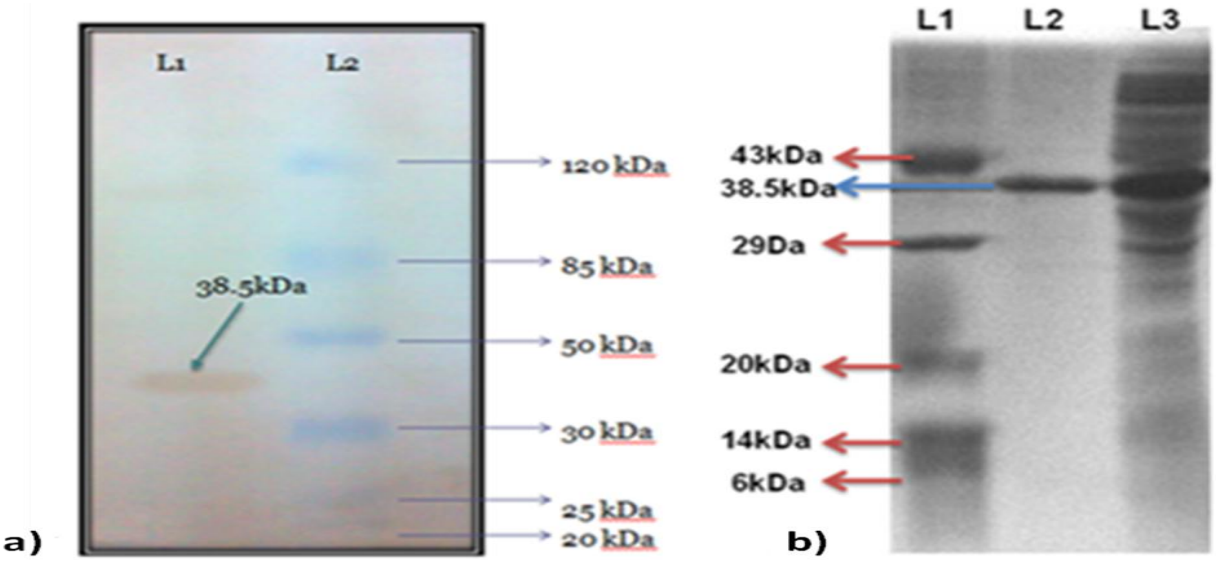

Figure 1. (a) Western blot, L1- purified OmpC protein, L2- prestained protein mol.wt. marker (Fermentas). **(b)** Purification of recombinant 38.5kDa OmpC protein by Ni-NTA affinity chromatography. L1- unstained protein mol.wt. marker (GeNei^{TM}) low range, L2- purified rOmpC protein, L3- total cell lysate.

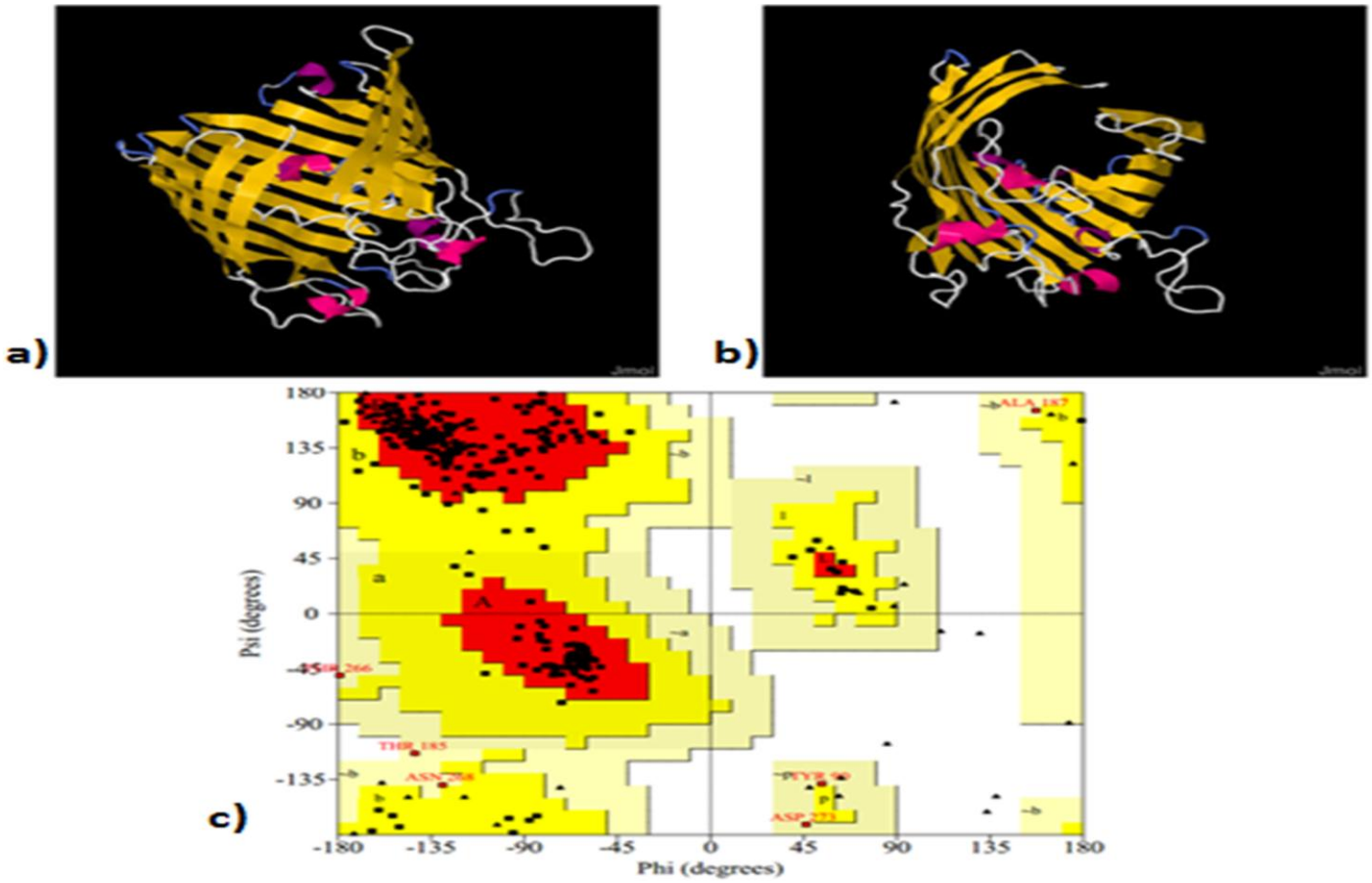

Figure 2. (a-c) The 3-Dimensional structure of OmpC porin protein predicted using EsyPred3d online resource and Jmol as visualization tool, showing major transmembrane β-sheet regions and exposed α-helical structures- front view and top view. (c) Ramachandran plot for OmpC protein generated by PBDsum for identifying the favourability of the protein.

Table 1. List of representative Receptors and their relevant interaction energies with the OmpC protein 3D structure.

PDB ID	Receptor molecule	Resolution Å	Docking energy E total (kcal/mol)
P01867 (IGG2B)	Ig gamma-2B chain C region. Igh-3	2.0	-818.31
7FAB	Crystal Structure of Human Immunoglobulin Fragment FAB. IGG1	2.0	-1296.89
1MAM	Crystal Structure of Monoclonal FAB specific for Brucella A cell wall. Igh-3	2.45	-763.87
1IGA	Model of IGA1 determined by Solution Scattering curve fitting and Homology modeling. IGH1		-8514.05
1IGY	Crystallographic structure of an intact IgG1 monoclonal antibody	3.2	-1753.32
1KCU	Crystal structure of Antibody PC287. Ig kappa chain C region	2.2	-893.53
1KCV	Crystal structure of antibody PV282.	1.8	-1167.93
3GJF	High Affinity T-cell receptor like antibodies. HLA-A, β-2-microglobulin	1.9	-853.33

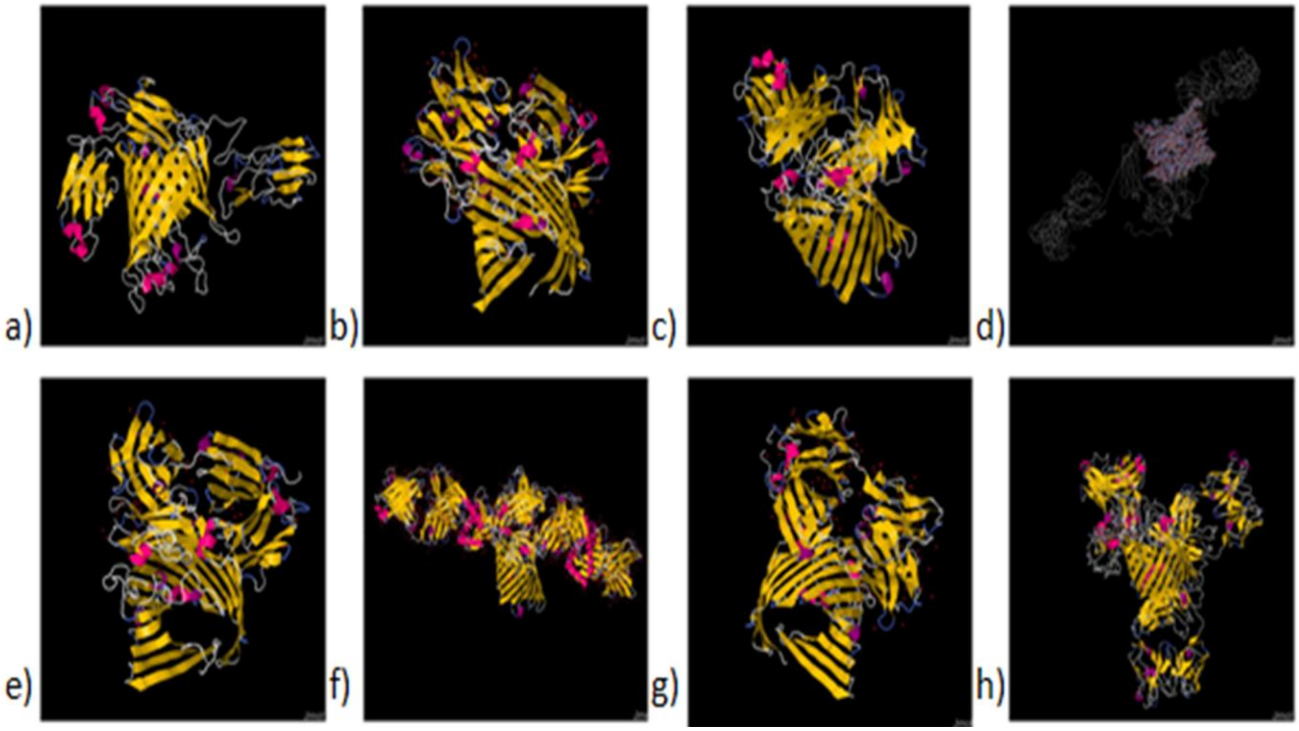

Figure 3. (a-h) Immune complex predictions using the eight 3D structures as given in Table 1.

Study of immunopotential of OmpC protein

The immunogenic potential of rOmpC was evaluated by western blotting and a sharp band was observed with anti Omp antisera indicating a strong humoral response of 38.5 kDa rOmpC in animal model as compared to total outer membrane proteins used for immunization (Figure 1a). In correspondence with the earlier *in silico* studies of *ompC* gene was done using Immune Epitope Database. The presence of many epitopes was revealed in the sequence including 13 B-cell epitopes, out of which eight were predicted to be strongly immunogenic, 14 MHC I and several MHC II epitopes (Jha et al., 2012). Further, eight major variable regions were found by multiple sequence alignment with the related sequences having high surface probability and B-cell recognition potential, indicating the high immunogenic potential of the putative protein (Jha et al., 2012). These *in silico* studies conducted earlier by us and western blot directed the animal trials for OmpC protein.

Assessment of immunogenic potential in poultry

A strong humoral immune response was elicited in immunized group. The mean of logarithm of reciprocal of

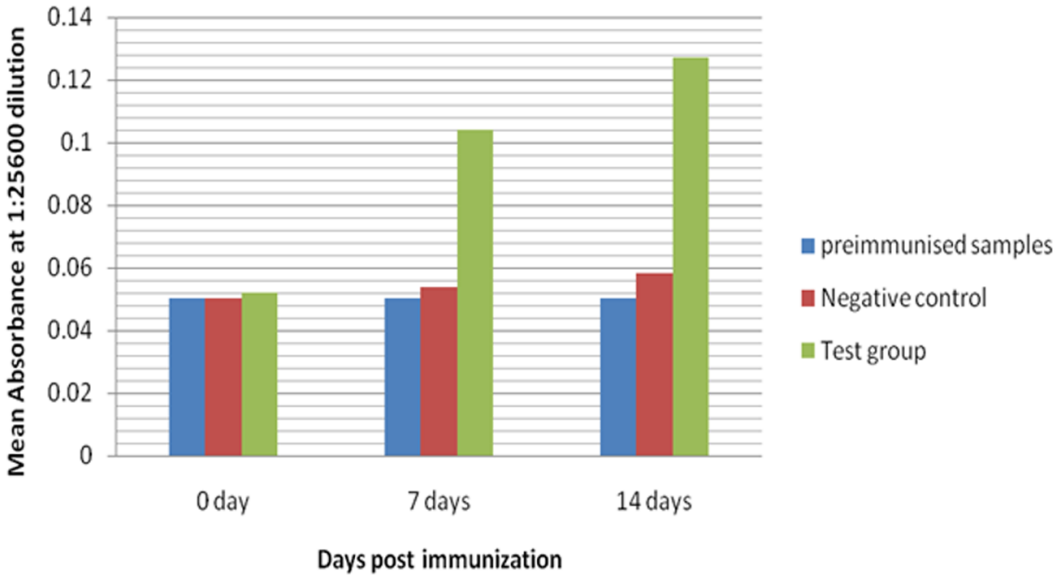

Figure 4. Comparative evaluation of recombinat OmpC antibody titres in immunized White Leghorn by ELISA, at different intervals (1:25600 dilution).

Table 2. Evaluation of the percentage of birds shedding the organism in immunized group as compared to control group.

Group	Days post challenge		
	0th day	5th day	10th day
Unimmunized control	0%	58%	83%
r-OmpC adjuvanted with saponin	0%	20%	33%

titre was estimated to be 4.40 ± 0.077 for 7th (1:29400) and 4.46 ± 0.06 for 14th day (1:32000) samples. The mean OD at a dilution of 1:25600 was calculated and the results were compared with that of control group (Figure 4). No statistically significant association was found in the humoral responses in control and vaccinated groups (p value = 0.064) revealing a strong significant antibody response generated in the vaccinated group as compared to control group.

Challenge studies

To assess the protective response generated against bacterial challenge, the immunized and control group was challenged 2 weeks post immunization. Birds in the immunized group did not show any apparent symptoms of Salmonellosis although the control group showed lethargic movements and decreased response to stimuli. In the control group, out of 15, 4 birds died at 0, 1, 3 and 4th day post challenge, although no mortality was observed in immunized group (Table 2). Cloacal swabs from all surviving birds were evaluated to estimate shedding. The *S.* Typhimurium virulent strain was identified in swabs samples using *Salmonella* specific PCR. The shedding percentage in control group was found to be 58 and 83% on 5th and 10th day, respectively, while in the case of immunized group it was 20 and 33% with no mortality. The fecal shedding of virulent strain was observed to be lower but not completely controlled in immunized birds than in unimmunized control. The association of immunization with rate of survival and with shedding was estimated by applying Chi-square test with Yate's correction. It was found that there was no significant association between immunization and the rate of survival of birds (p = 0.1071) (Supplementary 3). Similarly, no significant association was observed between immunization and shedding of bacteria (p = 0.3279) (Supplementary 4). Although, recombinant OmpC induced strong humoral response but fecal shedding of bacteria was slightly lowered and not effectively controlled. Persistence of infection was observed in some vaccinated birds. The humoral immune response developed by rOmpC immunization was found to be effective in controlling shedding in ~60% birds during challenge studies.

Porin proteins form β-barrels with long hydrophilic loops and short β-hairpin turns connecting strands on the external and periplasmic surfaces of the bilayer (Cowan and Rosenbusch, 1994). Their primary structure varies significantly among Gram-negative bacteria (Gerbl-Rieger et al., 1991), but amphiphilic β strands in the barrel are structurally conserved (Jeanteur et al., 1994). It was found that OmpC of *S.* Typhimurium folds in a similar way as OmpF of *E. coli* and OmpC of *S.* Typhi (Arockiasamy et al., 2004b; Kumar and Krishnaswamy, 2005). The

expression of OmpC during infection (Puente et al., 1989; Verma et al., 2009) and its capacity to display heterologous epitopes on the cell surface (Puente et al., 1995) make it an important candidate antigen with potential applications in immunology and vaccine design. Studies using *Salmonella* porin specific monoclonal antibodies (Muthukkaruppan et al., 1992) showed that *S.* Typhi OmpC is the major surface antigen with unique exposed epitopes. The immunogenicity of outer membrane proteins was evaluated in birds and was found to be significantly high (Meenakshi et al., 1999), indicating that systemic immunity may play a role in protection. Hamid and Jain immunized different groups of mice with selected proteins. Their ELISA results indicated that the protein elicits a significant humoral response and different levels of protection. Maximum survival was seen in the animals immunized with 49 kDa protein (100%), followed by 37 kDa (66.7%), 33 kDa (50%) and 15kDa (33.3%) (Hamid and Jain, 2008). Verma et al. (2009) evaluated the immunogenicity of the recombinant porins, in Swiss albino mice with three different adjuvants. In contrast to OmpF, the high titer ($p < 0.05$) of recombinant OmpC-specific IgG antibody was observed in mice immunized with aluminium hydroxide gel followed by Freund's adjuvant and montanide. The marked IgG responses in mice immunized with OmpC and OmpF supported the immunogenic nature of these recombinant porins and suggests that these may be used as good immunogens for vaccine studies (Verma et al., 2009). Another immunodominant surface protein of 37.81 kDa was immunologically characterized *in vivo* in chicks which induced homologous or heterologous protective efficacy against the infection of *Salmonella* serovars (Begum et al., 2008).

In the present study, although a strong humoral response was generated but the shedding of bacteria form fecal route was not completely eradicated, although significant reduction in mortality was observed. The generation of cell mediated response against *Salmonella* may be targeted in future studies by using adjuvants having depot formation property. Liposomes and ISCOMs have been reported to generate CTL induction and act as immunomodulators. These adjuvants may be used for providing the cell mediated immune response for obtaining better protection using r-Omp C as vaccine candidate.

Conclusions

Such results highlight the fact that the protein being a potent immunogen generates partially protective antibodies. These partially protective antibodies generated contribute in suppressing mortality along with slight reduction in fecal shedding of the pathogen in challenged birds. Although the antibodies may not participate in lowering zoonotic transmission, it may be hypothesized that they may play significant role in curbing mortality from virulent pathogen. Hence, it may be concluded that the protein OmpC, being immunogenic is partially protective in nature. The improvement in survival rate, suppression of physiological symptoms and reduction in fecal shedding signify the fact that the antibodies generated in the host system are partially protective. Therefore, OmpC protein may be considered as strongly immunogenic and partially protective. Further, the protective efficacy of the recombinant protein may be improved by focusing on specific epitopes and modulating the regions which improve protectivity through *in silico* experimentation. Furthermore, improvements such as new and advanced vaccine delivery systems like nano- drug delivery particles, ISCOMs, Liposomes etc which work as immunomodulators may be incorporated. These adjuvants may also be useful for providing cell mediated immune response which would further aid in enhancing the protective role of OmpC as a vaccine candidate.

Conflict of interests

The authors did not declare any conflict of interest.

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Use of simple sequence repeat (SSR) markers for screening blue disease resistance in cotton germplasm exchange

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Blue disease of cotton is an economically important disease of the crop first described from the Central African Republic and spread to other countries. Brazil and other South American countries record crop losses of up to 80% from infection but no cases of the disease have been reported in Tanzania. Resistance to the disease has been found in African germplasm and transferred to crop cultivars worldwide. Molecular markers linked to blue disease resistance genes have been identified presenting useful tools to identify resistant germplasm. All plants of three Tanzanian cotton cultivars (*Gossypium hirsutum L.*) UK91, UK08 and UKM08 showed resistance alleles for both the simple sequence repeat (SSR) (DC20027-202 bp) and single nucleotide polymorphisms (SNP) (NG0204310-C) markers but some plants of the Brazilian cultivars (*G. hirsutum L.)*; Ipê, Cedro, Aroeira and Araça lacked resistance alleles. The findings suggest the need for caution to be taken during introduction of exotic germplasm and recognize the value of resistance trait to susceptible Brazilian germplasm when breeding for blue disease resistance.

Key words: Cotton blue disease, cotton single nucleotide polymorphisms (SNPs), simple sequence repeat (SSR), resistant alleles in cotton.

INTRODUCTION

One of the diseases afflicting cotton that is of significant economic importance is cotton blue disease (CBD). CBD was first described in the Central African Republic in 1949 and has since been reported in various regions of Africa, Asia, and America (Brown, 2001; Correa et al., 2005; Junior et al., 2008). The disease is caused by a virus that belongs to the genus *Polerovirus* of the family Luteoviridae (Correa et al., 2005), which is transmitted by

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Abbreviations: SSR, simple sequence repeat; **CBD,** Cotton blue disease; **ECGA,** eastern cotton growing area; **BYDV,** barley yellow dwarf virus; **MAS,** marker assisted selection; **WCGA,** western cotton growing area.

cotton aphids (*Aphis gossypii* Glover) in a persistent circulative manner (Costa et al., 1997; Santos, 2001). Diseased plants are characterized by leaf rolling, vein yellowing, a moderate to severe stunting due to shortening of internodes, and dark green to bluish coloration of leaves (Brown, 2001). Despite CBD being first described in Africa, no cases of this disease have been reported from Tanzania although the vector (Cotton aphid, *Aphis gossypii*) has been reported in the Eastern Cotton Growing Area (ECGA) (Kabissa, 1993; Mrosso and Kabissa, 2000). Other members of Luteoviridae family, which affect cereals, have been also reported in Tanzania. Webby et al. (1993) reported the occurrence of Barley Yellow Dwarf Virus (BYDV). Lack of records of blue disease in Tanzania may potentially be due to the absence of the pathogen or presence/ use of resistant germplasm.

In Brazil and other South American countries, the disease poses serious problems for cotton production, capable of significantly reducing productivity of susceptible varieties by up to 80% if the vectors (cotton aphids) are not properly managed at the early growing stage (Silva et al., 2008). Losses of up to 1,500 kg per hectare of seed cotton have been reported in Brazil (Freire, 1998), where blue disease is the most important virus and a main concern for the breeding program. Generally, control of CBD is through breeding for resistance and control of cotton aphids using insecticides. Insecticides are expensive, harmful to the environment, and provide seasonal protection unlike use of resistant cultivars that offer a better but less used management option to control the disease (Fang et al., 2009). Therefore, use of resistant germplasm is one of the most economical and reliable methods in managing diseases in cotton. Naturally, occurring resistant parental germplasm is vital for development of resistant crop varieties. Identification of resistant germplasm is done either by screening artificially inoculated plants or screening for known molecular markers closely linked to resistance genes. Artificial inoculation requires controlled environments like greenhouses and growth chambers and can be time-consuming and costly, limiting the application of such phenotype-based resistance selection. The use of molecular markers in Marker Assisted Selection (MAS) unlike artificial inoculation can be performed at any development stage of the plant including on seeds at reasonable cost and with a fast turnaround of information compared to phenotype-based selection (Xiao et al., 2009).

Additionally, germplasm exchange can introduce genotypes susceptible to diseases in a new environment and screening prior to introduction is a challenge in the absence of the pathogen. The use of MAS provides an important tool in identifying resistance in the germplasm to be exchanged. A single gene controls resistance to CBD in cotton (Junior et al., 2008) and molecular markers associated with resistance gene are currently available for use in screening for resistant germplasm (Fang et al., 2009). Fang et al. (2009) conducted a study and identified SSR marker DC20027 that is closely linked to a cotton blue disease resistance locus. Based on the SSR marker they also identified three SNP markers NG0203671, NG0204310, NG0203481, which were tightly linked to the CBD resistance gene. Tanzania has a long standing breeding programme in the Western Cotton Growing Area (WCGA), which started at Ukiriguru in 1939. The focus of the programme has been to develop varieties that are high yielding; diseases and pest resistant; high ginning out turn and exhibit improved fibre properties.

The narrow genetic base of the cotton varieties in Tanzania (Lukonge et al., 2007) necessitates the need to introduce new and more diverse varieties from other countries for improvement. Brazil has a relatively broad genetic base of cotton varieties and a history of variety development by the governmental organizations since 1921 (Freire et al., 2011) including introduction of genetic material from different origins followed by crossing and selection (Gridi-Papp et al., 1991). Some of varieties have outstanding field performance and resistance to some diseases (Cia et al., 2008). This may serve as source of germplasm to improve the genetic base of Tanzanian cotton. Screening such germplasm for resistance to diseases prior to introduction of the same into breeding program is vital. The information on the resistance of Tanzanian cotton varieties to cotton blue disease is lacking and this is one of important diseases in Brazil. This study therefore, endeavored to screen the Tanzanian and Brazilian cotton cultivars (*Gossypium hirsutum L.*) for presence of markers closely associated with blue disease resistance gene using SSR and SNP markers.

MATERIALS AND METHODS

The study site

The growth experiments were setup at Lake Zone Agricultural Research and Development Institute (LZARDI) in the WCGA 02°42'S 33°01'E, about 30 km from Mwanza city in Misungwi District, Tanzania. Molecular marker analyses were conducted at Embrapa Rice and Beans (Santo Antonio de Goias, Brazil) and DNA Landmarks, Canada. LZARDI is an Agricultural research institute in Tanzania with the mandate for research on cotton and other crops in the WCGA.

Plant materials

Seven cotton cultivars (*G. hirsutum L.*) from Tanzania (UK91, UK08 and UKM08) and Brazil (Ipê, Cedro, Aroeira and Araça) were screened in this study. The Tanzanian cultivars are commercial varieties currently cultivated in the WCGA and were all developed LZARDI. The Brazilian cultivars were developed by Embrapa and are also commercially released varieties. Seeds of the test cultivars were sown in the screen house in a Randomized Complete Block Design at LZARDI. All the plants were tagged immediately after they were established.

Table 1. Primer sequences.

Primer		Sequence
DC20027	Forward primer	AATAAACCCTTCAGACAACAG
	Reverse primer	CTACCTAGTTTTGCATTATG
NG0204310	Forward primer	CCCCTGTTTACGAGGCTATCTATTC
	Reverse primer	GGTTTGGCCCAGTGACTAGAAG

Table 2. Proportions of plants with markers linked to Cbd resistance gene.

Variety	% of samples with resistance allele at DC20027 locus	% of samples with resistance allele at NG0204310 locus
UK91	100	100
UKM08	100	100
UK08	100	100
Araça	91	91
Cedro	91	91
Aroeira	83	83
Ipê	75	75

Leaf sampling for DNA isolation

Tissues for DNA isolation were obtained from young leaves of individually tagged Tanzanian and Brazilian cotton plants grown in the field three weeks after germination. Sampling was done by punching two discs of the same leaf into a single well of a 96-well plate. Five grams of silica gel were used to dry samples in the plate and replacement was made every till when the samples were satisfactorily dry. Seventeen (17) plants were sampled from each variety.

DNA isolation and quantification

For the SNP marker NG0204310, DNA isolation was performed using the DNA Landmarks' standard micro-extraction protocol at DNA Landmarks, Canada. DNA concentrations were measured using Hoechst dye and the quality of the DNA samples was checked on a 0.8% agarose gel (1 column per plate) prior to SNPs analyses. For the SSR marker DC20027, DNA isolation was done at Embrapa, Brazil. Briefly, 0.2 g of plant material was ground in mortar and pestle under liquid nitrogen to fine powder then added to preheated extraction buffer and mixed thoroughly. The extract was incubated at 65°C for 1 h, and mixed in every 20 min then cooled for 5 min and chloroform: isoamyl alcohol (24:1) mixture was added and vortexed for 30 min before centrifuging for 20 min at 3000 × *g*. The supernatant was then transferred to a fresh tube and same volume of ice-cold isopropanol added and tubes inverted several times. The tubes were then centrifuged for 30 min at 3000 × *g*. The pellet was washed with 70% ethanol, dried and dissolved in 1 × TE buffer. DNA concentration was checked using Nanodrop 2000 (Thermo Scientific, USA) then diluted to 10 ng/µL for PCR amplification.

SNP genotyping

The SNP genotyping and analysis using the markers NG0204310 was carried out using the Sequenom Mass ARRAY® Typer 4.0 software.

SSR Amplification and scoring

SSR amplification for the marker DC20027 was done at Embrapa. The PCR mixture (5 µl) contained 1 µl template DNA (10 ng/ µl), 2.5 µl of 2× QIAGEN PCR Master Mix (HotStarTaq DNA Polymerase, PCR Buffer and dNTP Mix), 0.5 µl of Q solution (PCR additive that facilitates amplification of difficult templates by modifying the melting behavior of DNA), 0.06 µl of forward and reverse primers and 0.88 µl of sterile double distilled water (Table 1). Amplification was performed in 0.2 ml (each tube) thin walled PCR plates (96 tubes / plate) in a thermal cycler (Applied Biosystems). The DNA was first activated at 95°C for 15 min and amplification temperatures were: denaturation 94°C for 1 min and 30 s, then annealing at 55°C for 1 min followed by extension at 72°C for 1 min, for 40 cycles. The PCR products were genotyped using Genetic Analyzer (Applied Biosystems Inc.) and scoring was done using GeneMapper software. Deltapal was included as positive control (resistant) and FM966 as negative control (susceptible).

RESULTS

Deltapal which is known to be resistant and was used as positive control had 202 bp at SSR DC20027 and C at NG0204310 while FM966 (susceptible) had 200 bp on SSR DC20027 and T at NG0204310 locus. All samples for varieties from Tanzania (UK91, UK08 and UKM08) showed resistance alleles for both SSR (DC20027-202bp) and SNP (NG0204310-C) markers. Some of plants of the cultivars from Brazil, which is, Ipê, Cedro, Aroeira and Araça lacked resistance alleles, as shown in Table 2. The two markers presented coincident results, that is, the potentially susceptible plants lacked both the

SSR and the SNP linked to the locus, and the potentially resistant plants showed the SSR and the SNP linked to it.

DISCUSSION

Cotton breeding in Tanzania is challenged by reduced genetic diversity of *G. hirsutum* as observed among the 26 studied varieties by Lukonge et al. (2007). This is currently being addressed by studying the potential consequences of introduction of more diverse cultivated cotton varieties from other countries to enable improvement of the available germplasm. CBD caused by Cotton leaf roll dwarf virus of the genus *Polerovirus* of the family Luteoviridae is a serious problem in cotton growing countries in the world especially in South America. Brazil reported yield losses up to 80% in susceptible varieties (Silva et al., 2008). CBD spreads very fast and is difficult to control. In this study, all plants of the Tanzanian cultivars tested indicated the presence of alleles linked to the CBD resistance gene at SSR DC20027 and SNP NG0204310 loci. Screened Brazilian cultivars on the other hand registered a mixture of both marker alleles present in resistant and susceptible plants. These findings are important to the cotton-breeding programme in Tanzania as they provide the baseline information on the presence of resistance markers in local germplasm and the efforts to sustain this trait should be considered. The fact that all plants that had resistance marker on SSR DC20027 locus confirmed the presence of the same trait on the SNP NG0204310 locus and the presence of markers linked to resistance gene in all tested plants of the Tanzanian cultivars as observed in this study indicates their potentiality for such trait which could be confirmed by artificial inoculation.

The results from this study conform to prior information given Lukonge (Pers. Comm.) on the lack of reported cases of the CBD in the WCGA in the recent years. The results further suggest the possibility of using this valuable germplasm in breeding for CBD resistance in areas where the disease causes significant losses once confirmation on their resistance is done. Thus, screening of exotic germplasm for CBD should always be done before incorporation of exotic germplasm into local breeding programmes. However, it is yet to be established whether absence of cases of the disease is due to absence of pathogen or resistant germplasm. It is believed that CBD resistant varieties across the world have been developed through crosses with African germplasm. For instance, in 1980s, South American countries particularly Brazil and Argentina introduced the African germplasm to combat this disease (Royo et al., 2003). It is thus likely that the partial resistance traits observed in the Brazilian germplasm in this study originated from African germplasm. Findings of this study further complement the observation made by Fang et al. (2009) who screened different cotton varieties for the presence of markers linked to CBD and found that most of varieties from Africa and South America had the markers for resistance.

The observed scenario that Brazilian germplasm had mixture of both resistance markers and susceptible ones to the disease suggests the need to make such populations homozygous for the trait. This could be possible through use of marker assisted selection. Individual plants of Brazilian cultivars that presented resistance markers at both loci can however be considered for multiplication and introduction into local breeding program to improve the local cotton production. Basing on the results of this study, it can be concluded that there is partial resistance in the population of Brazilian cultivars screened and so selection for breeding using such cultivars should consider using plants with resistance trait to CBD and marker assisted selection could be a useful tool to achieve such selection.

Conflict of interests

The authors did not declare any conflict of interest.

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Antibiosis in *Ascia monuste orseis* Godart (Lepidoptera: Pieridae) caused by kale genotypes

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***Ascia monuste orseis* (Lepidoptera: Pieridae) is one of the main insect pests of kale. The study was done to identify kale varieties resistant to *A. monuste orseis* by the antibiosis resistance mechanism. Kale genotypes (26) were evaluated in experiments performed at the Laboratory of Agricultural Entomology of Goiano Federal Institute - Campus Urutaí. A completely randomized experimental design with 50 replicates was used. The biological parameters evaluated were (a) larval stage: development time, instars, viability and larval weight 15 days after hatching; (b) pupal stage: development time, weight of 24-h-old pupae, viability; (c) larvae-adult stage: development time and viability. The genotypes Gigante I-915 and Pires 1 de Campinas have antibiosis resistance. Gigante I-915 caused high larval mortality and Pires 1 de Campinas resulted in low larval and pupal viability of *A. monuste orseis*.**

Key words: *Brassica oleracea* L. var. *acephala*, Brassicaceae, Great Southern White, host plant resistance, integrated pest management (IPM).

INTRODUCTION

Kale (*Brassica oleracea L. var acephala D.C*) is an important vegetable for human consumption because it is rich in minerals and essential vitamins (Ferreres et al., 2007). This plant is originally from the Mediterranean and Southwestern Europe, occurring from the north to south of England (Vaughan and Geissler, 1997).

Among the insect pests that occur in kale, the caterpillar *Ascia monuste orseis* (Lepidoptera: Pieridae) is particularly relevant due to its frequent occurrence and the severe defoliation it causes (Schlick-Souza et al., 2011; Baldin et al., 2014). The insect *A. monuste orseis* is mainly observed in plants of the family: Brassicaceae, including narrow leaved pepperwort (*Lepidium ruderale*), broccoli (*Brassica oleracea* var. *italica*), wild mustard (*Sinapis arvensis*), wild radish (*Raphanus raphanistrum*), cabbage (*Brassica oleracea* var. *capitata*), cauliflower (*Brassica oleracea* var. *botrytis*) and Chinese cabbage (*Brassica rapa* var. *pekinensis*) as well in forest crops

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(Chamberlin and Kok, 1986; Lasota and Kok, 1989; Kok and Acosta-Martinez, 2001; Pratissoli et al., 2007).

Infestation by *A. monuste orsei*sis is primarily controlled by periodical application of chemical insecticides (Kuhar et al., 2003). These products may cause serious problems such as residues in food, elimination of natural enemies, toxicity to handlers and selection of insecticide-resistant populations (Roel et al., 2000).

The use of alternative methods is a promising strategy for controlling pests in vegetable plants because it reduces the amount of insecticides applied and the levels of residues in food. Therefore, the use of kale varieties resistant to insects becomes important to control pests of this crop (Fancelli and Vendramim, 1992; Boiça Juniior et al., 2011; Schlick-Souza et al., 2011; Baldin et al., 2014).

Host-plant resistance is a control method within the precepts of integrated pest management (IPM), especially to reduce the pest population density to a level below the economic threshold with no impact on the agro-ecosystem and no additional costs for the farmer. Host-plant resistance also has persistent effects during the phenological cycle of crops and is compatible with other methods (Eigenbrode and Trumble, 1994; Seifi et al., 2013; Sousa et al., 2014). Resistance in Brassicaceae can manifest by antibiosis, which disrupts the insect's biology and reduces its abundance and the damage caused by the pest, and/or by antixenosis that affects the insects' behavior and is usually expressed as feeding or oviposition non-preference in resistant plants (Painter, 1951; Panda, 1979; Lara, 1991; Smith, 2005).

There are few studies of resistance of kale to *A. monuste orseis*, and their analyses are based on biological data of the pest development in hosts and not on the host plant characteristics (Schlick-Souza et al., 2011). The genotypes Manteiga de Jundiaí, Comum, Arthur Nogueira 1, Manteiga de Ribeirão Pires I-2446, Manteiga de Ribeirão Pires I-2620 and Tronchuda Portuguesa exhibited oviposition non-preference (antixenosis) resistance. The genotypes Japonesa, Pires 1 de Campinas, Roxa I-919 and Manteiga de São Roque I-812 exhibited feeding non-preference in *A. monuste orseis* (Schlick-Souza et al., 2011).

Chemical, morphological and physical characteristics of the Brassicaceae are involved in those resistance mechanisms to insects (Farnham and Elsey, 1995; Renwick and Kimberly, 1999; Ulmer et al., 2002; Thuler et al., 2007; Vendramin and Guzzo, 2009; Baldin and Beneduzzi 2010; Schlink-Souza et al., 2011).

The present study evaluates the antibiosis resistance mechanism in kale genotypes by determining the biological parameters of *A. monuste orseis* caterpillars under laboratory conditions.

MATERIALS AND METHODS

Seedlings of the kale genotypes were obtained from the Laboratory of Plant Resistance to Insects (LPRI), School of Agricultural and Veterinary Sciences, University of São Paulo States, Municipality of Jaboticabal-São Paulo, and were transplanted to the field at the Goiano Federal Institute-Campus Urutaí–Goias States.

The cultivation practices were performed following the recommendations for kale crops and irrigation was applied as needed using a conventional sprinkler irrigation system (Filgueira, 2008). The following genotypes were studied: Manteiga de Mococa, Manteiga de Jundiaí, Manteiga de Tupi, Pires 2 de Campinas, Vale das Garças, Crespa de Capão Bonito, Couve Arthur Nogueira 1, Couve Arthur Nogueira 2, Hortolândia, Orelha de Elefante, Crespa I-918, Manteiga I-1811, Manteiga de Ribeirão Pires I-1811, Manteiga de Ribeirão Pires I-2620, Verde Escura, Pires 1 de Campinas, Verde Claro, Manteiga de São José, Manteiga de Monte Alegre, Roxa I-919, Couve Comum, Manteiga de São Roque I-1812, Manteiga de Jaboticabal, Geórgia 1, Geórgia 2 and Gigante I-915.

Insect rearing and maintenance

Egg masses were collected from kale plants, transported to the Laboratory of Agricultural Entomology (T 25 ± 2°C, 70 ± 10% RH and 12 h photophase), and placed in Petri dishes (14 cm diameter) containing moistened filter paper until the larvae hatched.

The newly hatched larvae were transferred to rearing cages (50 cm height x 30 cm diameter), sealed with tulle fabric secured with the aid of an elastic band. The bottom of the rearing cages was lined with paper towels to absorb the moisture from the excrement. Initially, 50 first-instar larvae were placed in each cage. Kale leaves were placed in pots (250 mL) containing water to maintain turgor pressure and were renewed daily or according to the larval development.

The pupae were placed in hatching cages (300 mL plastic cups), the bottom of which was covered with paper towel and the top was sealed with tulle fabric secured with the aid of an elastic band. The hatching cages were moistened daily to avoid possibly drying of pupae. After emergence, the adults were released in the field to ensure copulation, oviposition and egg collection.

Antibiosis test

To assess biological parameters, newly hatched *A. monuste orseis* larvae were placed in Petri dishes (14 cm diameter) containing a leaf disc (10 cm diameter) of each kale genotype and were kept in these containers from the larval to pupal stage. The pupae were placed in the same hatching cages used for *A. monuste orseis* rearing. Sexual identification and longevity of adults were assessed after their emergence.

The following biological parameters were evaluated: (a) larval stage: development time, instars, viability and larval weight 15 days after hatching; (b) pupal stage: development time, weight of 24-h-old pupae, viability and sex ratio; (d) larval-adult stage: development time and viability. A completely randomized experimental design with 26 treatments and 50 replicates was used. The treatments consisted of the different kale genotypes.

Statistical analysis

The biological parameters of *A. monuste orseis* were assessed by analysis of variance (ANOVA). For analysis, the original data were $(x+0.5)^{1/2}$ transformed. Means were compared using the Scott-Knott test at a 5% probability level (Winer et al., 1991). The statistical analysis was performed using the software SISVAR (Ferreira, 2011). Cluster analysis and Euclidean distance analysis were performed as a dissimilarity measure using the software Statistica

Table 1. Development time (mean±standard error) of the larval stages of *Ascia monuste orseis* (Lepidoptera: Pieridae) in kale genotypes (25°C, 70% RH and 12 h photophase).

Genotype (G)	Development time (days)				
	1° instar	2° instar	3° instar	4° instar	5° instar
Manteiga de Mococa	2.03±0.04b	2.91±0.07a	2.95±0.04a	3.49±0.22b	5.65±0.19a
Manteiga de Jundiaí	1.65±0.10c	1.97±0.02d	1.82±0.25b	3.09±0.10b	3.61±0.19d
Manteiga de Tupi	2.02±0.02b	1.99±0.00d	1.55±0.23b	2.52±0.22c	3.70±0.19d
Pires 2 de Campinas	2.02±0.02b	3.00±0.00a	2.83±0.21a	3.25±0.19b	3.82±0.11d
Vale das Garças	1.49±0.22d	2.05±0.05d	3.42±0.19a	3.17±0.21b	4.56±0.23c
Crespa de Capão Bonito	1.20±0.20f	2.50±0.22c	2.20±0.20b	2.49±0.22c	5.14±0.16b
Couve Arthur Nogueira 1	2.04±0.04b	2.01±0.01d	2.24±0.19b	4.51±0.22a	5.68±0.10a
Couve Arthur Nogueira 2	1.06±0.06f	2.16±0.11d	2.25±0.19b	3.14±0.22b	4.84±0.10b
Hortolândia	1.00±0.00f	2.19±0.12d	2.25±0.32b	3.20±0.20b	4.08±0.10c
Orelha de Elefante	1.10±0.11f	2.80±0.09b	3.05±0.20a	3.22±0.19b	4.24±0.15c
Crespa I-918	1.08±0.09f	2.52±0.12c	2.28±0.19b	2.71±0.20c	4.05±0.06c
Manteiga I-1811	2.03±0.04b	2.25±0.11d	2.26±0.20b	2.55±0.23c	4.23±0.19c
Manteiga de Ribeirão Pires I-1811	2.01±0.01b	2.77±0.10b	2.30±0.12b	3.33±0.21b	5.00±0.00b
Manteiga de Ribeirão Pires I-2620	1.30±0.14e	2.07±0.08d	2.11±0.20b	2.77±0.14c	3.69±0.20d
Verde Escura	1.69±0.09c	3.06±0.04a	2.29±0.09b	3.02±0.02c	4.64±0.27d
Pires 1 de Campinas	1.30±0.05e	3.06±0.12a	2.13±0.06b	3.04±0.32c	4.11±0.16c
Verde Claro	2.75±0.14a	3.09±0.09a	2.57±0.20b	2.81±0.20c	4.13±0.13c
Manteiga de São José	1.99±0.00b	3.10±0.10a	2.63±0.20a	3.48±0.22b	5.56±0.11a
Manteiga de Monte Alegre	2.06±0.06b	2.07±0.07d	2.28±0.19b	3.26±0.19b	3.06±0.07e
Roxa I-919	2.01±0.01b	3.02±0.03a	2.25±0.21b	2.55±0.23c	4.13±0.13c
Couve Comum	2.05±0.05b	2.64±0.19b	3.18±0.12a	3.57±0.24b	5.35±0.16a
Manteiga de São Roque I-1812	1.78±0.05c	2.31±0.20c	2.16±0.12b	2.86±0.10c	4.36±0.13c
Manteiga de Jaboticabal	1.52±0.02d	2.49±0.22c	2.35±0.12b	2.62±0.23c	4.06±0.06c
Geórgia 1	1.13±0.08f	2.01±0.02d	2.00±0.00b	2.72±0.13c	5.05±0.06b
Geórgia 2	1.73±0.06c	2.97±0.16a	2.17±0.12b	3.00±0.15c	4.14±0.14c
Gigante I-915	1.56±0.03d	2.22±0.10d	2.20±0.20b	2.68±0.00c	-2
F (G)	25.15**	13.56**	5.18**	4.95**	21.55**
C.V. (%)	11.64	10.12	17.24	14.52	7.50

Means followed by the same letter in the columns do not differ statistically based on Skott Knott test. **Significant at 1% probability level. [2]Caterpillars fed with these genotypes do not complete their cycle (no variance).

version 7.0 to separate the kale genotypes according to their resistance level to *A. monuste orseis*.

RESULTS AND DISCUSSION

Significant differences were observed in the development time of each instar and in the total life cycle of *A. monuste orseis* fed on the different kale genotypes (Table 1 and Figure 1). The first instar of *A. monuste orseis* larvae fed with the genotype Verde Claro had the longest development time (2.75 days), whereas the first instar of insects reared on the genotypes Hortolândia, Couve Arthur Nogueira 2, Crespa I-918, Orelha de Elefante, Geórgia 1 and Crespa de Capão Bonito exhibited the shortest development times (1.0-1.20 days).

Second instar larvae fed with the genotypes Manteiga de Mococa, Pires 2 de Campinas, Verde Escura, Pires 1 de Campinas, Verde Claro, Manteiga de São José, Roxa I-919 and Geórgia 2 showed the longest development times (2.91 to 3.10 days). The opposite was observed for the second instar of insects fed with the genotypes Manteiga de Jundiaí, Manteiga de Tupi, Vale das Garças, Couve Arthur Nogueira 1, Couve Arthur Nogueira 2, Hortolândia, Manteiga I-1811, Manteiga de Ribeirão Pires I-2620, Manteiga de Monte Alegre, Geórgia 1 and Gigante I-915, whose development times ranged from 1.97-2.25 days.

Insects fed with the genotypes Manteiga de Mococa, Pires 2 de Campinas, Vale das Garças, Orelha de Elefante, Manteiga de São José and Couve Comum had the longest development time at the third instar stage. For the 4th instar, insects fed with the genotype Couve Arthur Nogueira 1 had the longest development time. The genotypes Manteiga de Mococa, Couve Arthur Nogueira

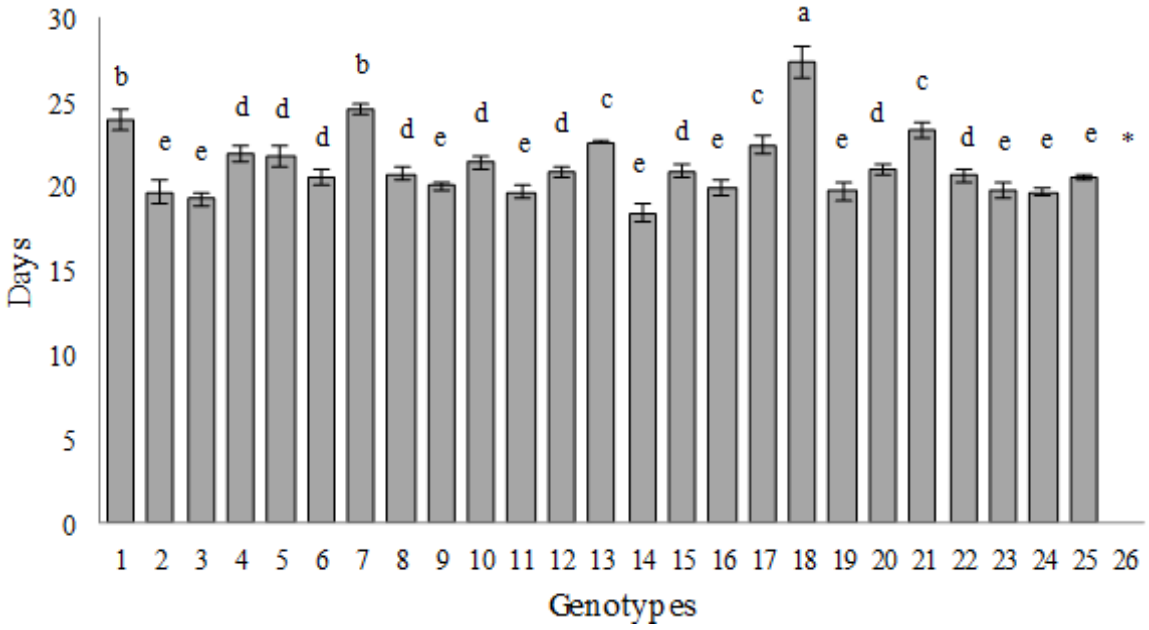

Figure 1. Development time (mean ± standard error) of the total life cycle of *Ascia monuste orseis* (Lepidoptera: Pieridae) in kale genotypes (grown at 25°C, 70% RH and14 h photophase). Urutaí, Goiás States, Brazil. Means followed by the same letter in the columns do no differ statistically by the Scott Knott test. [2]Caterpillars fed with these genotypes did not complete their life cycle (no variance). F (treatment) = 17.72** and C. V(%) = 4.97. Genotypes: **1.** Manteiga de Mococa; **2.** Manteiga de Jundiaí; **3.** Manteiga de Tupi; **4.** Pires 2 de Campinas; **5.** Vale das Garças; **6.** Crespa de Capão Bonito; **7.** Couve Arthur Nogueira 1; **8.** Couve Arthur Nogueira 2; **9.** Hortolândia; **10.** Orelha de Elefante; 11. Crespa I-918; **12.** Manteiga I-1811; **13.** Manteiga de Ribeirão Pires I-1811; 14. Manteiga de Ribeirão Pires I-2620; 15. Verde Escura; **16.** Pires 1 de Campinas; **17.** Verde Claro; **18.** Manteiga de São José; **19.** Manteiga de Monte Alegre; **20.** Roxa I-919; **21.** Couve Comum; **22.** Manteiga de São Roque I-1812; **23.** Manteiga de Jaboticabal; **24.** Geórgia 1; **25.** Geórgia 2; and **26.** Gigante I-915. [*]Caterpillars fed with these genotypes do not complete their cycle (no variance).

1, Manteiga de São José and Couve Comum resulted in the longest development time for the 5th instar, and insects fed with the genotype Gigante I-915 did not complete their cycle.

In general, throughout the larval stage, *A. monuste orseis* caterpillars that fed on the genotypes Manteiga de Mococa, Couve Comum, Manteiga de São José, Couve Arthur Nogueira 1, Manteiga de Ribeirão Pires I-1811 and Verde Claro showed the longest larval stage. The slowest larval development in these genotypes may have occurred due to the presence of glucosinolates or other factors that cause such resistance (Thuler et al., 2007; Baldin et al., 2014).

The presence of glucosinolates in brassicas has been previously related to resistance of these plants to *Pieris rapae* (Linnaeus, 1758) and *Plutella xylostella* (Linnaeus, 1758) as reported by Renwick and Kimberly (1999) and Thuler et al. (2007). A longer larval development time in *A. monuste orseis* is characteristic of kale genotypes that present antibiosis and/or antixenosis type resistance (Smith, 2005; Baldin et al., 2014).

Plant resistance may also be related to the waxiness of the leaf surface and the levels of secondary compounds such as sinigrin and alkane in Brassicaceae (Ulmer et al. (2002). Antibiosis type resistance was observed by Fancelli and Vendramim (1992) in the genotype Manteiga de Tupi. It has been observed that a longer larval development time in insects that fed with the genotypes Arthur Nogueira 1, Cabocla, Japonesa and Manteiga de Mococa (Baldin et al., 2014). However Verde-escura, Crespa de Capão Bonito, Couve de folhas Manteiga 900 Legítima Pé Alto, Gigante I-915 and the genotype Manteiga Ribeirão Pires I-2446 reduced the larval weight. Gigante I-915 produced high larval mortality. Pupae reared in the genotype Pires 1 de Campinas did not reach the adult stage and the genotypes Japonesa and Arthur Nogueira 1 prolonged the development time from egg to adult of *A. monuste orseis*. These data corroborate the results of the current study in which the larval stage of *A. monuste orseis* caterpillars that fed on the genotype Manteiga de Mococa was extended.

A. monuste orseis caterpillars fed with the genotypes Manteiga de São José, Couve Arthur Nogueira 1, Manteiga de Mococa and Couve Comum had the longest life cycles, with mean values ranging from 23.39 to 27.39 days (Figure 1).

Table 2. Larval and pupal weight and viability (mean±standard error) of *Ascia monuste orseis* (Lepidoptera: Pieridae) in kale genotypes (25°C, 70% RH and 14% photophase).

Genotype (G)	Weight (mg)		Viability (%)	
	Larval	Pupal	Larval	Pupal
Manteiga de Mococa	344.2±3.87a	337.6±1.78c	45.00±0.95g	43.20±1.88i
Manteiga de Jundiaí	311.2±4.97b	294.6±2.79f	63.40±2.34d	72.47±1.47d
Manteiga de Tupi	318.2±7.89b	309.4±4.13e	70.40±2.20c	76.56±1.61c
Pires 2 de Campinas	302.8±5.05c	345.2±1.83b	90.40±1.36b	97.10±0.51a
Vale das Garças	312.4±5.55b	312.2±1.66e	76.00±1.38c	67.60±1.36e
Crespa de Capão Bonito	297.0±3.98c	333.8±5.47c	93.12±1.09b	97.28±0.43a
Couve Arthur Nogueira 1	307.2±5.58b	295.8±3.63f	63.40±0.68d	60.40±0.51f
Couve Arthur Nogueira 2	295.4±5.27c	353.4±5.18a	51.00±1.67f	52.42±0.48g
Hortolândia	298.2±4.87c	292.2±1.66f	96.44±0.54a	97.09±0.59a
Orelha de Elefante	296.4±2.54c	338.6±2.99c	95.54±1.00a	97.07±0.49a
Crespa I-918	290.8±3.80c	284.2±2.03g	93.78±0.44b	95.44±0.50a
Manteiga I-1811	287.0±4.82d	344.6±1.91b	67.82±1.45c	66.26±0.82e
Manteiga de Ribeirão Pires I-1811	269.4±4.08e	288.2±3.60g	96.74±0.36a	96.97±0.52a
Manteiga de Ribeirão Pires I-2620	289.0±4.72c	315.4±2.38e	20.40±1.29h	14.08±0.74m
Verde Escura	283.2±5.81c	330.0±1.30d	65.35±0.90d	65.08±3.07e
Pires 1 de Campinas	298.6±1.47c	298.6±0.81f	02.60±0.40i	02.40±0.51n
Verde Claro	304.4±7.35c	336.8±1.46c	22.32±0.83h	28.02±1.56l
Manteiga de São José	245.8±6.41g	308.0±1.60e	67.82±1.43c	65.84±0.82e
Manteiga de Monte Alegre	292.0±3.00c	290.2±1.85g	63.98±1.68d	55.60±0.75g
Roxa I-919	297.0±5.21c	338.6±1.40c	70.03±0.96c	73.64±0.78d
Couve Comum	262.0±2.02f	325.4±1.96d	61.52±1.36d	45.34±0.91i
Manteiga de São Roque I-1812	236.2±2.67g	329.6±0.51d	55.60±0.97e	48.73±1.43h
Manteiga de Jaboticabal	223.4±3.71h	345.2±2.31b	62.22±1.52d	58.88±2.13f
Geórgia 1	223.6±3.47h	353.4±3.39a	22.76±0.68h	34.20±0.66j
Geórgia 2	215.0±4.81h	349.0±4.82b	94.44±0.29a	86.00±0.55b
Gigante I-915	203.6±1.81i	-2	-2	-2
F (G)	57.51**	65.57**	451.74**	498.92**
C.V. (%)	3.72	1.95	4.27	4.17

Means followed by the same letter in the columns do not differ statistically according to Skott Knott test. **Significant at 1% probability level. [2]Caterpillars fed with these genotypes do not complete their cycle (no variance).

According to Lara (1991), the extension of the insect life cycle reduces the number of generations over time, thus reducing the population size and increasing the pest exposure to natural enemies. Therefore, the genotypes Manteiga de São José, Couve Arthur Nogueira 1, Manteiga de Mococa and Couve Comum, which extended the life cycle of *A. monuste orseis*, are promising alternatives for the integrated management of this pest in the field because these genotypes showed resistance to *A. monuste orseis*. The durations of the life cycle of insects fed the above mentioned genotypes are similar to those reported by Baldin et al. (2014), who observed a total life cycle of 22.8 and 33.3 days for caterpillars fed on the genotypes Verde Escura and Japonesa, respectively.

Regarding the larval and pupal weight and viability, both of these biological parameters showed significant differences for insects fed with the different kale genotypes (Table 2). *A. monuste orseis* caterpillars fed on the genotype Manteiga de Mococa had the highest larval weight (344.2 mg). Conversely, caterpillars reared on the genotype Gigante I-915 had the lowest larval weight (203.6 mg) and did not reach the pupal stage. The genotypes Geórgia 1 and Couve Arthur Nogueira 2 produced the greatest pupal weight (353.4 mg), while insects fed with the genotypes Crespa I-918, Manteiga de Ribeirão Pires I-1811 and Manteiga de Monte Alegre had the lowest pupal weights, with mean values ranging from 284.2-290.2 mg.

The results obtained by Baldin et al. (2014) are similar to those found in the present study. The authors observed that *A. monuste orseis* caterpillars fed on the genotype Gigante I-915 did not reach the pupal stage. This pattern demonstrates the antibiosis or antixenosis

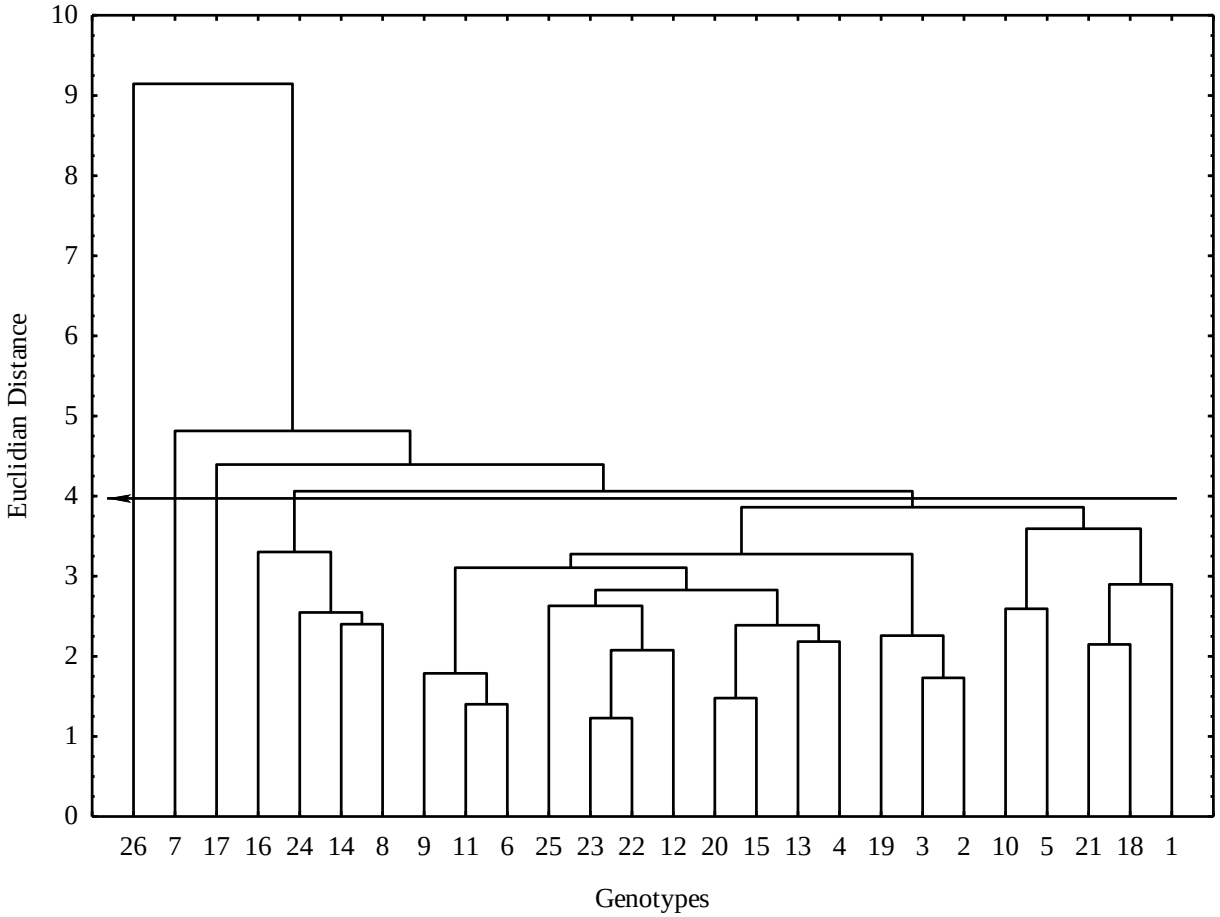

Figure 2. Dendrogram based on biological parameters of *Ascia monuste orseis* (Lepidoptera: Pieridae) in twenty-six kale genotypes. The method of agglomeration was used with UPGMA dissimilarity measure of Euclidean distance. Urutaí, Goiás States, Brazil. Genotypes: **1.** Manteiga de Mococa; **2.** Manteiga de Jundiaí; **3.** Manteiga de Tupi; **4.** Pires 2 de Campinas; **5.** Vale das Garças; **6.** Crespa de Capão Bonito; **7.** Couve Arthur Nogueira 1; **8.** Couve Arthur Nogueira 2; **9.** Hortolândia; **10.** Orelha de Elefante; **11.** Crespa I-918; **12.** Manteiga I-1811; **13.** Manteiga de Ribeirão Pires I-1811; **14.** Manteiga de Ribeirão Pires I-2620; **15.** Verde Escura; **16.** Pires 1 de Campinas; **17.** Verde Claro; **18.** Manteiga de São José; **19.** Manteiga de Monte Alegre; **20.** Roxa I-919; **21.** Couve Comum; **22.** Manteiga de São Roque I-1812; **23.** Manteiga de Jaboticabal; **24.** Geórgia 1; **25.** Geórgia 2; and **26.** Gigante I-915.

type resistance of this kale genotype to *A. monuste orseis* caterpillars. This resistance can be associated with chemical or physical factors (Ulmer et al., 2002; Thuler et al., 2007; Baldin et al., 2014).

The insects fed with the genotypes Hortolândia, Orelha de Elefante, Manteiga de Ribeirão Pires I-1811 showed the highest larval and pupal viability. In contrast, the genotypes Manteiga de Ribeirão Pires I-2620 and Pires 1 de Campinas generated the lowest viability.

Studies have shown that the genotype Pires I de Campinas produced feeding non-preference in *A. monuste orseis* (Schlick-Souza et al., 2011). The genotype Pires I de Campinas, which caused the lowest larval and pupal viability in the present study, has compounds that exert a deterrent effect and manifest different types and levels of resistance to *A. monuste orseis*.

Based on the hierarchical cluster analysis, there were diferences among the genotypes, which were divided in five groups according to their similarity levels (Figure 2): Group 1 (Gigante I-915); Group 2 (Couve Arthur Nogueira 1); Group 3 (Verde Claro); Group 4 (Pires 1 de Campinas, Geórgia 1, Manteiga de Ribeirão Pires I-2620 and Couve Arthur Nogueira 2); and Group 5 (Hortolândia, Crespa I-918, Crespa de Capão Bonito, Geórgia 2, Manteiga de Jaboticabal, Manteiga de São Roque, Manteiga I-1811, Roxa I-919, Verde Escura, Manteiga de Ribeirão Pires I-1811, Pires 2 de Campinas, Manteiga de Monte Alegre, Manteiga de Tupi, Manteiga de Jundiaí, Orelha de Elefante, Vale das Garças, Couve Comum, Manteiga de São José and Manteiga de Mococa).

By setting the Euclidean distance at 4.0, the following

division of the genotypes into distinct groups according to their levels of resistance is suggested: Gigante I-915, Couve Arthur Nogueira 1, Verde Claro, Pires 1 de Campinas, Geórgia 1, Manteiga de Ribeirão Pires I-2620 and Couve Arthur Nogueira 2 moderately resistant (MR); Hortolândia, Crespa I-918, Crespa de Capão Bonito, Geórgia 2, Manteiga de Jaboticabal, Manteiga de São Roque, Manteiga I-1811, Roxa I-919, Verde Escura, Manteiga de Ribeirão Pires I-1811, Pires 2 de Campinas, Manteiga de Monte Alegre, Manteiga de Tupi and Manteiga de Jundiaí susceptible (S); Orelha de Elefante, Vale das Garças, Couve Comum, Manteiga de São José and Manteiga de Mococa highly susceptible (HS).

Conclusions

The genotypes Gigante I-915 and Pires 1 de Campinas have antibiosis resistance. Gigante I-915 caused high larval mortality and Pires 1 de Campinas resulted in low larval and pupal viability of *Ascia monuste orseis*.

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Estimation of genetic diversity between three Saudi sheep breeds using DNA markers

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The genetic variation of Najdi, Harri and Awassi breeds of Saudi sheep prevailing in Raniah province of Makka district were assessed and compared to Sudanese Desert sheep using random amplified polymorphic DNA polymerase cahin reaction (RAPD-PCR) technique. Five primers successfully amplified distinguishable 40 bands with an average of 96% polymorphism revealing that Saudi sheep breeds possess the needed genetic variation required for further genetic improvement. The resulted dendrogram showed that, there are two main separate clades. The Desert sheep is genetically distant and appeared as out-group from the Saudi sheep breeds. The first main clade included all of the Najdi individuals and only two individuals from Harri breed. While, the second main clade comprised two subgroups, the first one included individuals from Harri breed and the second included both Harri and Awassi individuals. The cluster analysis shows that Najdi breed is genetically different from both Harri and Awassi and that some Harri individuals showed genetic closeness to Awassi. The present study will help to clarify the image of the genetic diversity of these local Saudi sheep breeds in Raniah province and should be followed by further studies using advanced DNA markers and all available breeds in the kingdom to get the precise estimation of the phylogeny of these local genetic resources.

Key words: Dendrogram, biodiversity, Sudanese sheep, random amplified polymorphic DNA (RAPD), Saudi sheep.

INTRODUCTION

The population of sheep in the Kingdom of Saudi Arabia is about 5.2 million head (Saudi Ministry of Agriculture, 2011). In Raniah Province of Makka district alone, there are 250000 heads of sheep (Al Faraj, 2003). Harri and Najdi sheep breeds are Saudi local reflect good adaptive traits to the local environmental conditions and meet the

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Abbreviations: RAPD, Random amplified polymorphic DNA; **PCR,** polymerase chain reaction.

Saudi consumer needs. Najdi is the principal native sheep breed in the eastern province of Saudi Arabia (Aljumaah et al., 2014). Awassi (also known as Naemi), sheep have been exported from its origin in east of the Mediterranean to more than 30 countries in all continents of the world including KSA (Galal et al., 2008). It is known for its good milk production (Al-Atiyat and Aljumaah, 2014). Indigenous sheep breeds are valuable source of genetic material due to adaptation to local harsh environmental conditions, nutritional fluctuations and resistances to diseases and parasites (Nsoso et al., 2004; Galal et al., 2008). Unfortunately, accelerated decline of biodiversity worldwide was reported and 20% of the domestic animal breeds are at risk of extinction (FAO, 2000; Kunene et al., 2009). Particularly for sheep, it is estimated that 180 sheep breeds (14%) are extinct (Cardellino, 2004; FAO, 2007). There is terrible risk that most breed may perish before they have been exclusively recognized and exploited. Conservation and maintenance of animal genetic biodiversity of local breeds will facilitate the effective management of farm animal genetic resources. There is a need to genetically re-evaluate these breeds to assess the existing population structure and differences which would serve to facilitate the future conservation programs. On the basis of microsatellite data, considerable genetic differentiation was recently reported in Saudi Najdi sheep (Musthafa et al., 2012). The first step in the conservation and utilization of indigenous sheep breeds is characterization and evaluation of genetic diversity which is a prerequisite for improving any species (FAO, 2007; Bjornstad and Roed, 2001; Notter, 1999).

The traditional phenotypic characterization can now be complemented by molecular markers and sophisticated statistical techniques for data analysis. Random amplified polymorphic DNA (RAPD) is a polymerase chain reaction (PCR) based fingerprinting technique that amplifies random DNA fragments with single short primers of arbitrary nucleotide sequence under low annealing stringency (Williams et al., 1999; Awad et al., 2010). The RAPD markers have been described as a simple and easy method to use for estimation of genetic variability among breeds or species (Kumar et al., 2008; Ruane, 2000). The objective of the present study was to utilize the RAPD technique to characterize three Saudi sheep breeds (namely Awassi, Harri and Najdi) in Raniah province and to estimate the genetic diversity within and between these breeds and Sudanese Desert Sheep as outbreed.

MATERIALS AND METHODS

Study area

The study area pertains to Raniah Province of Makkah district (12° 30 N, 42° E) in the west part of Saudi Arabia extending over 62,000 km^2. Raniah Province lies about 870 km south- west to Riyadh, 380 km west to Taif and 150 km north to Bishah. The province has similar meteorological and ecological attributes with the rest of the Arabian Peninsula. It is characterized by hot arid desert type climate, with average annual rainfalls of 90 mm, maximum temperature between 34 and 45°C in summer and between zero and 20°C in winter with an average relative humidity of 22% (Al Faraj, 2003).

Animals

Full mouthed unrelated females were randomly selected from three Saudi sheep breeds, namely, Awassi (Naeimi), Harri and Najdi to serve as blood donors. Twenty individuals were sampled from Najdi, 14 from Harri and five from Awassi. Blood was also collected from three Sudanese desert sheep to serve as outbreed for comparison.

Genomic DNA extraction

Blood samples from Jugular vein were collected from full mouthed unrelated female. At least 5 ml blood sample was drawn from the vein in the neck of each animal and collected in EDTA vacutainers. The blood was gently mixed with anticoagulant and kept at -20°C. Genomic DNA was extracted from peripheral blood lymphocytes according to instructions of blood DNA preparation kit (Jena Bioscience, Germany).

PCR amplification

The PCR amplification was performed in a 25 µl reaction volume, using Promega PCR master mix according to the instructions by the manufacturer with 30 Pmol from each of the primers: Initial denaturation at 94°C for 2 min, followed by 35 cycles consisting of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, extension at 72°C for 2 min and a final extension at 72°C for 2 min. Amplified products were electrophoresed on 1.5% agarose gel at constant voltage and 1X TBE for approximately 1.5 h. They were visualized by staining with ethidium bromide and photographed under ultraviolet light and molecular weights were estimated using 1 Kbp DNA ladder.

Scoring and statistical analysis

PCR products were scored across the lanes as variables. The presence of a band of amplified DNA was scored as (1) and absence as (0). The data generated was used for calculation of similarity matrix based on Nei and Li (1979). Very faint bands were excluded from the analysis. Similarity coefficients were utilized to generate a phylogenetic tree (dendrogram). Pairwise genetic distance between individuals were calculated by the percentage disagreement method. These data were used in cluster analysis with the unweighted pair-group method using arithmetic averages (UPGMA), in which samples were grouped based on their similarity with the aid of statistical software package STATISTCA- version 9 (StatSoft Inc., 2009).

RESULTS AND DISCUSSION

Information on genetic relationships in livestock within and between species has several important applications for genetic improvement and breeding programmes (Appa Rao et al., 1996). Comprehensive knowledge of the existing genetic variability is the first step for the

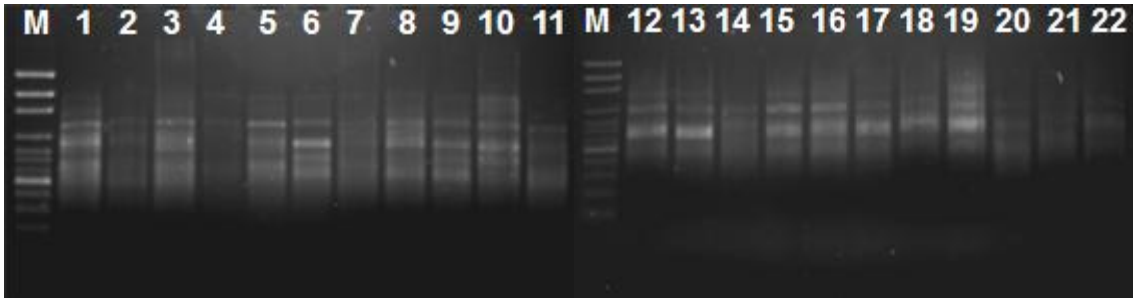

Figure 1. RAPD fingerprint generated from Najdi (1-20) and Harri (21-22) sheep breeds using OPL20 primer.

Table 1. The sequences of primers used and their polymorphic bands among three Saudi and one Sudanese sheep breeds.

Primer	Sequence of primer (5'-3')	Total number of bands	Number of polymorphic bands	Number of monomorphic bands	Polymorphism (%)
OPL-11	ACGATGAGCC	7	7	0	100
OPAL-20	AGGAGTCGGA	9	9	0	100
OPAL-15	AGGGGACACC	9	9	0	100
OPE-18	GGACTGCAGA	9	9	0	100
OPB-5	TGCGCCCTTC	6	5	1	83.33
Total		40	39	1	
Average		8	7.8	0.2	96.67

RAPD primers from Operon Technologies Inc. (Alameda Calif., USA).

conservation and exploitation of domestic animal biodiversity. Therefore, the objective of this study was to evaluate the genetic diversity of sheep breed in Raniah province of Saudi Arabia based on RAPD analysis. Five, out of 17 tested primers successfully amplified polymorphic bands between the different sheep breeds. The amplified PCR product of DNA showed identical band patterns with similar intensity (Figure 1). Out of the total distinguished 40 amplified fragments, 39 were polymorphic with an average of 7.8 bands. The maximum number of fragments (9 bands) was produced by three primers with 100% polymorphism, while the minimum numbers of fragments were produced by primer OPB-5 with 83.33% polymorphism (Table 1). The very high polymorphic rate (96.67%) indicated that the studied sheep breed possess the needed genetic variation for potential future preservation and breed development. Although, all studied population of Saudi Najdi, Hbsi, Arb, and Naemi sheep had substantial levels of genetic variation, but Najdi sheep had the highest gene diversity (Aljumaah et al., 2014).

Table 2 shows the genetic distance between individuals of the four goat breeds. Individuals designated with numbers from 1 to 20 are Najdi, from 21 to 34 are Harri, from 35 to 39 are Awassi and from 40 to 42 are Sudanese Desert Sheep. The highest genetic distance (0.53) was found between Najdi individual (N14) and the three Desert sheep individuals (Desert sheep-1-3). On the other hand, the least genetic distance (0.0) was found between Najdi individuals N1 and N2 and also between the two Desert sheep individuals (40 and 42). Genetic distance value of 0.0 reflects very high similarity between any two individuals. The distance measure between two clusters is calculated from the formula: *D=1-C*; where, D is the distance and C the correlation between object clusters. If objects are highly correlated, they will have a correlation value close to 1 and genetic distance value close to zero. Therefore, highly correlated clusters are nearer to the bottom of the dendrogram. Object clusters that are not correlated have a correlation value of zero and a corresponding genetic distance value of 1. Objects that are negatively correlated will have a correlation value of minus1 and genetic distance of 2.

As shown in Figure 2, the resulted dendrogram constructed from RAPD-PCR data showed that the Desert Sheep is genetically distant and appeared as out-group to the Saudi goat breeds. The result also shows that, there are two main separate clades. Most of the individuals that belong to the same breed were clustered together. The first main clade included Najdi individuals (N1-N20) and only two individuals from Harri breed. While the second main clade comprises two subgroups, in which the first subgroup contained only individuals from Harri breed (H3-H9 individuals). On the other hand, the second subgroup of the second main clade included Harri and Awassi individuals. The cluster analysis shows

Table 2. Matrix of RAPD dissimilarity among three Saudi sheep and Desert sheep breeds based on Nei and Lei coefficients

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
1	0.00																				
2	**0.00**	0.00																			
3	0.15	0.15	0.00																		
4	0.23	0.23	0.18	0.00																	
5	0.30	0.30	0.15	0.08	0.00																
6	0.30	0.30	0. 15	0.08	**0.00**	0.00															
7	0.38	0.38	0.23	0.15	0.08	0.08	0.00														
8	0.40	0.40	0.25	0.18	0.15	0.15	0.13	0.00													
9	0.30	0.30	0.20	0.13	0.15	0.15	0.18	0.15	0.00												
10	0.35	0.35	0.20	0.18	0.15	0.15	0.13	0.15	0.15	0.00											
11	0.33	0.33	0.23	0.15	0.18	0.18	0.20	0.18	0.13	0.13	0.00										
12	0.25	0.25	0.15	0.18	0.20	0.20	0.23	0.25	0.20	0.15	0.18	0.00									
13	0.28	0.28	0.28	0.25	0.28	0.28	0.25	0.33	0.23	0.23	0.25	0.18	0.00								
14	0.28	0.28	0.23	0.30	0.28	0.28	0.30	0.33	0.28	0.28	0.30	0.28	0.15	0.00							
15	0.38	0.38	0.38	0.35	0.38	0.38	0.30	0.38	0.33	0.38	0.35	0.33	0.20	0.15	0.00						
16	0.35	0.35	0.30	0.33	0.35	0.35	0.28	0.30	0.30	0.25	0.23	0.20	0.18	0.18	0.13	0.00					
17	0.40	0.40	0.30	0.33	0.30	0.30	0.28	0.35	0.35	0.30	0.33	0.25	0.18	0.23	0.23	0.15	0.00				
18	0.33	0.33	0.28	0.30	0.33	0.33	0.30	0.38	0.38	0.38	0.35	0.28	0.25	0.25	0.15	0.13	0.18	0.00			
19	0.40	0.40	0.40	0.28	0.30	0.30	0.28	0.35	0.35	0.35	0.33	0.35	0.28	0.33	0.23	0.20	0.20	0.18	0.00		
20	0.38	0.38	0.38	0.30	0.33	0.33	0.30	0.38	0.38	0.38	0.35	0.38	0.30	0.35	0.25	0.23	0.23	0.15	0.08	0.00	
21	0.38	0.38	0.38	0.25	0.28	0.28	0.20	0.28	0.28	0.28	0.25	0.33	0.25	0.30	0.20	0.18	0.23	0.20	0.13	0.10	0.00
22	0.33	0.33	0.33	0.15	0.23	0.23	0.20	0.23	0.23	0.28	0.30	0.28	0.25	0.30	0.25	0.23	0.23	0.20	0.18	0.20	0.15
23	0.38	0.38	0.43	0.30	0.38	0.38	0.30	0.38	0.33	0.33	0.35	0.38	0.30	0.35	0.35	0.33	0.33	0.40	0.33	0.40	0.30
24	0.30	0.30	0.35	0.23	0.30	0.30	0.23	0.30	0.25	0.25	0.28	0.30	0.23	0.28	0.28	0.25	0.30	0.33	0.25	0.28	0.18
25	0.40	0.40	0.35	0.33	0.35	0.35	0.28	0.35	0.30	0.30	0.33	0.30	0.23	0.33	0.33	0.30	0.30	0.28	0.35	0.33	0.28
26	0.40	0.40	0.35	0.28	0.30	0.30	0.23	0.25	0.30	0.30	0.33	0.35	0.23	0.33	0.28	0.25	0.30	0.23	0.25	0.23	0.23
27	0.38	0.38	0.38	0.30	0.38	0.38	0.30	0.38	0.33	0.33	0.35	0.38	0.25	0.30	0.30	0.28	0.28	0.30	0.33	0.30	0.30
28	0.48	0.48	0.48	0.30	0.38	0.38	0.30	0.38	0.38	0.38	0.35	0.43	0.30	0.35	0.25	0.28	0.28	0.30	0.23	0.30	0.25
29	0.48	0.48	0.48	0.30	0.38	0.38	0.30	0.38	0.38	0.38	0.35	0.43	0.30	0.35	0.25	0.28	0.28	0.30	0.23	0.30	0.25
30	0.35	0.35	0.40	0.23	0.30	0.30	0.28	0.25	0.30	0.35	0.28	0.40	0.23	0.28	0.23	0.25	0.30	0.28	0.25	0.28	0.23
31	0.35	0.35	0.45	0.28	0.35	0.35	0.43	0.35	0.35	0.45	0.43	0.45	0.33	0.38	0.43	0.45	0.40	0.43	0.35	0.43	0.43
32	0.33	0.33	0.28	0.10	0.18	0.18	0.25	0.28	0.23	0.28	0.20	0.28	0.30	0.35	0.40	0.38	0.33	0.35	0.33	0.35	0.35
33	0.33	0.33	0.28	0.10	0.18	0.18	0.25	0.28	0.23	0.28	0.25	0.28	0.30	0.35	0.40	0.38	0.33	0.35	0.28	0.35	0.35
34	0.33	0.33	0.33	0.15	0.23	0.23	0.25	0.33	0.28	0.28	0.30	0.28	0.30	0.40	0.40	0.38	0.38	0.35	0.28	0.35	0.35
35	0.30	0.30	0.25	0.18	0.25	0.25	0.23	0.30	0.30	0.30	0.33	0.25	0.33	0.38	0.33	0.30	0.35	0.23	0.25	0.28	0.33
36	0.40	0.40	0.35	0.18	0.25	0.25	0.23	0.20	0.25	0.30	0.33	0.30	0.38	0.48	0.43	0.40	0.40	0.38	0.30	0.38	0.33
37	0.40	0.40	0.35	0.18	0.25	0.25	0.23	0.25	0.25	0.30	0.33	0.30	0.38	0.48	0.43	0.40	0.40	0.33	0.30	0.38	0.33
38	0.33	0.33	0.33	0.20	0.28	0.28	0.25	0.33	0.33	0.33	0.35	0.33	0.40	0.50	0.45	0.43	0.43	0.35	0.33	0.35	0.35
39	0.35	0.35	0.30	0.18	0.25	0.25	0.28	0.35	0.30	0.30	0.33	0.30	0.33	0.38	0.38	0.35	0.35	0.28	0.25	0.28	0.33
40	0.35	0.35	0.40	0.28	0.35	0.35	0.33	0.40	0.40	0.40	0.38	0.35	0.38	**0.53**	0.43	0.40	0.35	0.33	0.25	0.18	0.23
41	0.40	0.40	0.45	0.33	0.40	0.40	0.38	0.45	0.45	0.45	0.43	0.40	0.38	**0.53**	0.43	0.40	0.35	0.33	0.25	0.18	0.28
42	0.35	0.35	0.40	0.28	0.35	0.35	0.33	0.40	0.40	0.40	0.38	0.35	0.38	**0.53**	0.43	0.40	0.35	0.33	0.25	0.18	0.23

Table 2. Contd.

22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42
0.00																				
0.25	0.00																			
0.18	0.13	0.00																		
0.28	0.23	0.15	0.00																	
0.23	0.28	0.20	0.15	0.00																
0.25	0.15	0.13	0.13	0.13	0.00															
0.25	0.15	0.18	0.23	0.18	0.10	0.00														
0.25	0.15	0.18	0.23	0.18	0.10	0.00	0.00													
0.23	0.33	0.25	0.30	0.15	0.23	0.18	0.18	0.00												
0.28	0.28	0.35	0.40	0.25	0.28	0.23	0.23	0.20	0.00											
0.25	0.35	0.33	0.38	0.28	0.25	0.25	0.25	0.23	0.23	0.00										
0.25	0.30	0.33	0.38	0.28	0.25	0.20	0.20	0.23	0.18	0.05	0.00									
0.25	0.25	0.28	0.33	0.23	0.25	0.20	0.20	0.28	0.18	0.15	0.10	0.00								
0.23	0.33	0.30	0.30	0.20	0.23	0.23	0.23	0.25	0.30	0.18	0.13	0.13	0.00							
0.18	0.28	0.25	0.30	0.20	0.28	0.23	0.23	0.25	0.20	0.23	0.18	0.13	0.15	0.00						
0.18	0.28	0.25	0.25	0.25	0.28	0.23	0.23	0.30	0.25	0.23	0.18	0.13	0.15	0.05	0.00					
0.25	0.20	0.28	0.28	0.23	0.25	0.25	0.25	0.33	0.28	0.25	0.20	0.10	0.13	0.13	0.13	0.00				
0.23	0.38	0.35	0.35	0.25	0.28	0.28	0.28	0.30	0.30	0.18	0.13	0.13	0.10	0.20	0.20	0.18	0.00			
0.28	0.43	0.30	0.35	0.35	0.38	0.38	0.38	0.35	0.50	0.38	0.38	0.38	0.30	0.35	0.35	0.33	0.30	0.00		
0.33	0.48	0.35	0.40	0.35	0.38	0.38	0.38	0.35	0.50	0.38	0.38	0.38	0.30	0.40	0.40	0.38	0.30	0.05	0.00	
0.28	0.43	0.30	0.35	0.35	0.38	0.38	0.38	0.35	0.50	0.38	0.38	0.38	0.30	0.35	0.35	0.33	0.30	0.00	0.05	0.00

that Najdi breed is genetically different from both Harri and Awassi, with maximum distance from Desert sheep breed. Also, individuals from the same breed are genetically close to each other. However, some individuals from Harri (H11, H12, H13) showed genetic closeness to Awassi. The close kinship between Harri and Awassi might suggest some past crossing between these two geographically close populations.

In a study aimed to characterize genetic constitution of Awassi, Harri and Habsi Saudi sheep, using random amplified polymorphic DNA (RAPD) technique, the highest homogeneity was observed within Harri breed followed by Habsi and Awassi breeds (40 and 24.2%, respectively) (Sabir et al., 2013). The genetic structure of Saud sheep population including Najdi, Hbsi, Arb, and Naemi was investigated using microsatellite revealing substantial genetic variability, with average heterozygosity range of 0.759 to 0.811 (Aljumaah et al., 2014). The genetic characterization, however, should be a continuous process of surveying and monitoring of the existing indigenous breeds.

Conclusion and recommendation

The very high polymorphic rate (96.67%) indicated that the studied sheep breed possess the needed genetic variation for further potential future preservation and breed development. The result from this study shows that Najdi breed is genetically different from both Harri and Awassi and that some individuals from Harri showed genetic closeness to Awassi. The present study will help to clarify the image of the genetic diversity of these local Saudi sheep breeds and should be followed by further studies using large number of animals from different geographical regions in the kingdom to get the precise estimation of the phylogeny of these local genetic resources.

Conflict of interests

The authors did not declare any conflict of interest.

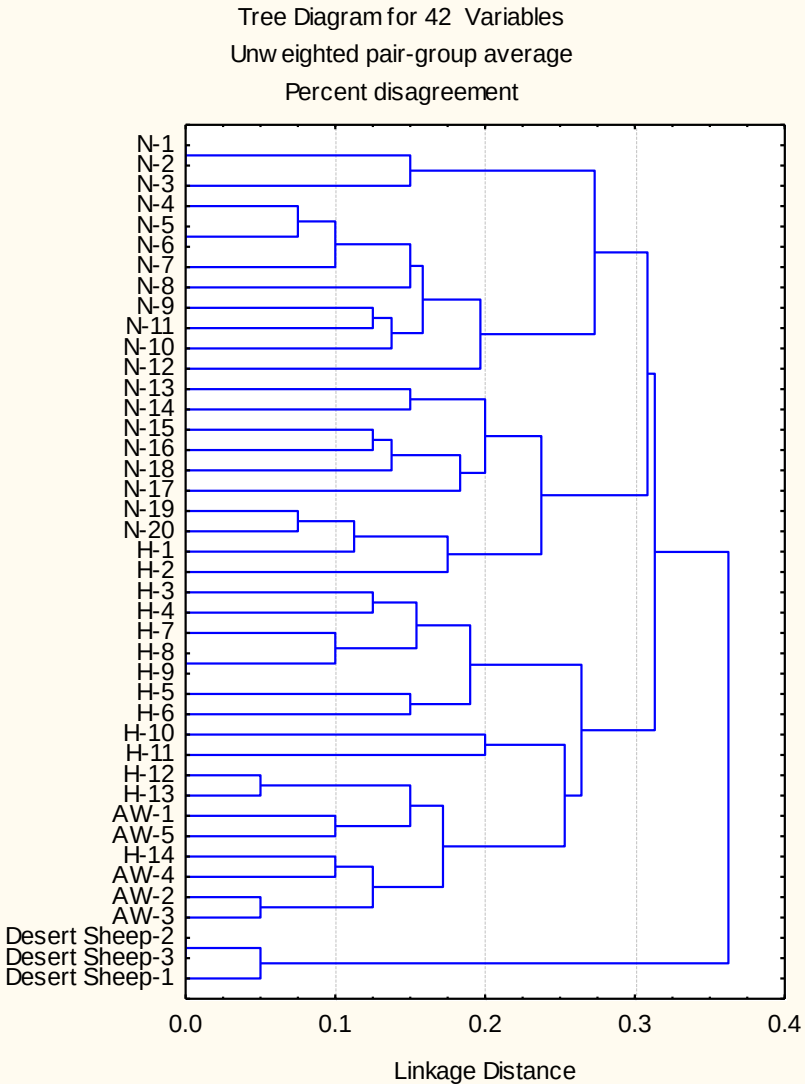

Figure 2. Phylogenic tree showing relationships among the four sheep breeds obtained by RAPD-PCR analysis using five primers. Individuals designated with N are Najdi; with H are Harri and with A are Awassi sheep breed.

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Full Length Research Paper

Determination of lactic acid bacteria in Kaşar cheese and identification by Fourier transform infrared (FTIR) spectroscopy

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Lactic acid bacteria (LAB) arise in Kaşar cheese, an artisanal pasta filata cheese produced in Turkey from raw milk without starter addition or pasteurized milk with starter culture. In this study, 13 samples of Kaşar cheese that were produced from raw milk were used as reference materials. LAB were characterized by using phenotypic, API and Fourier transform infrared (FTIR) spectroscopy methods. One hundred and fifty-seven (157) strains were isolated from 13 cheese samples, and identification test was performed for 83 strains. At the end of the study, a total of 22 *Lactococcus* sp., 36 *Enterecoccus* sp. and 25 *Lactobacillus sp.* were determined in the isolated strains by phenotypic identification. After identification with FTIR spectroscopy and statistical analysis, expected results were not taken by FTIR spectroscopy due to higher correlation (> % 99) obtained with more than one reference culture. Fewer number of reference strains was a limitation of the analysis. Therefore, identification should be made with more reference bacteria in the FTIR analysis and should also be supported with molecular techniques.

Key words: Kaşar cheese, lactic acid bacteria, identification, Fourier Transform Infrared (FTIR).

INTRODUCTION

Fourier transform infrared (FTIR) spectroscopy is used for characterization of many microorganisms. It is useful at the serogroup, species and genus level, data acquisition is faster than in PAGE and other techniques and preparation is simple and quick. FTIR spectroscopy and cluster analysis of bacterial cells is a promising tool not only for identification and classification purposes based upon bacterial IR spectra alone, but also as an additional aid to support conventional methods for clarifying relationships, especially within poorly classified taxa (Helm et al., 1991). Phenotypic and biochemical methods such as morphological and physiological properties, API CHL50 kit are useful at genus-species level but they are insufficient at sub species level for characterization of LAB (Kıran and Osmanağaoğlu, 2011). Molecular genetic techniques, such as randomly

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amplified polymorphic DNA analysis PCR-based methods have been successfully used to identify LAB at the subspecies level but it is difficult to adapt them for their use in routine laboratories due to their high costs and the requirement for highly skilled personnel (Kirschner et al., 2001). It is important to be able to identify and characterize LAB in the dairy industry with rapid, reliable and cheap methods (Amiel et al., 2000).

Kaşar is a semi-hard "pasta filata" (plastic curd) cheese which is produced by being boiled and kneaded after curd of the cheese is acidified (Üçüncü, 2008). Kaşar is one of the most produced and consumed cheese type in Turkey and in some of Balkan and European countries (with different names like Kaşkaval, Kassari, Kachkawaj) (Halkman and Halkman, 1991). Kaşar is produced in Turkey from raw milk without starter addition or pasteurized milk with addition starter culture. Therefore, Kaşar cheese produced from raw milk is fermented by lactic acid bacteria originated from the milk. The number of systematic studies aiming determination of starter culture in Kaşar cheese is very few (Akyüz, 1978; Tunail, 1978; Halkman and Halkman, 1991). The use of the right starter cultures is essential and is important to produce Kaşar cheeses with a constant high quality.

The main purpose of the present study was to compare biochemical identification methods with FTIR cluster method for identification of LAB isolated from Kaşar cheese. Also, the FTIR spectra of the isolates were compared to a spectrum library to obtain FTIR identification. Two methods were applied in this study in order to evaluate the relevance and mutual agreement between methods, and the capacity of each method to accurately identify different LAB species present in Kaşar cheese.

MATERIALS AND METHODS

Isolation of lactic acid bacteria from cheese samples and identification tests

A total of 13 Kaşar cheese samples supplied from various factories were used as reference materials under laboratory conditions. The reference strains for the characterization of the isolates from samples with FTIR spectroscopy were provided by Izmir Institute of Technology (IIT), Department of Food Engineering. Biochemical characterization was performed in order to identify isolates using carbohydrates. BD BBL ™ Crystal ™ Gram-Positive ID kits were used for the isolates that were determined as cocci. BioMerieux API 50 CHL (Ref. 50410 to 10 × 10 ml) test kits were used for the isolates that were determined as bacilli. Appropriate dilutions of cheese samples were prepared using the isolation of bacteria, and were plated in agar culture media. Isolation of *Lactococcus* sp. was performed by plating M17 (Merck) medium at 28°C for 24 to 48 h. While *Lactobacilli* sp. was isolated by plating MRS agar (Merck) medium at 30°C for 48 h, isolation of *Enterecoccus* sp. was carried out by using NB (Merck) medium at 37°C for 24 to 48 h at incubation (Tunail et al., 2001).

Morphological characteristics, catalase activities, litmus milk reductions and gram reactions of the isolates were performed as the physiological analyzes. Biochemical tests (growing such conditions 10, 15, 45°C, 6.5% NaCl, 4% NaCl, pH 9.2 to 9.6, 0.1 to 0.3% metylen blue reduction, Voges-Proskauer reaction, forming ammoniac from arginine, citrate reduction, 0.04% tellurite reduction, forming gas from glocuse and sugar fermentation tests) were carried out according to the Sherman classification (Sandine et al., 1962; Cowan and Steel, 1966; Harrigan and McCance, 1966; Tunail, 1978; Sürmeli, 1979; Tunail and Köşker, 1986; Salminen and Wright, 1993; Holt et al., 1994; Tunail et al., 2001). API 20 was applied for isolation of *Enterecoccus* sp. and *Lactococcus* sp. After the basic analysis, sugar and API50CHL tests were conducted for lactobacilli (Tunail et al., 2001).

Identification of lactic acid bacteria with FTIR analysis

After growing the isolates in the appropriate media, they were centrifuged at 5000 r.p.m. The pellet was washed and later lyophilized according to procedures previously described by Irudayaraj et al. (2002), Kuleaşan and Başyiğit Kılıç (2007) and Başyiğit Kılıç (2009).

Preparation of samples for FTIR spectroscopy

Ten milligram of lyophilized cultures was crushed together with approximately 300 mg of KBr in agate mortar. The mixture was transferred into 10 mm diameter disks and placed under 10 tons/cm² pressures for 10 min. The disks were analyzed by FTIR (Perkin Elmer, Spectrum 100). Spectra samples between 4000 and 450 cm^{-1} wavelengths were taken in the analysis. Each sample was scanned 64 times at 4 cm^{-1} resolution. The study was conducted with two parallels (Başyiğit Kılıç, 2009).

Evaluation of absorbance data

After reading the absorbance values obtained from the range of 4000 to 450 cm^{-1} wavelengths, they were analyzed by the use of the Statistical Package for the Social Sciences. Similar rates of isolates were determined with the aid of Pearson correlation product moment coefficient at regions of 3000 to 2800, 1500 to 1400, 900 to 700 cm^{-1} wavelengths in order to identify and separate strains. Hierarchical cluster analysis was applied to the isolates whose similarity rates were over 99% and dendogram was drawn with Ward's algorithm (Naumann et al., 1990).

RESULTS AND DISCUSSION

Results of phenotypic analysis

A total of 157 isolates were obtained from 13 cheese samples. Physiological and biochemical analysis were further conducted with 83 isolates in terms of morphological characteristics, catalase activities, and gram reactions. Biochemical tests were carried out based on Sherman classification. Cocci that could grow under conditions such 10, 15, 45°C, 6.5% NaCl and pH 9.6 were identified as enterococci. The rest of the cocci strains were evaluated as lactococci.

Taking these criteria and their phenotypic characteristics into account, 36 of 83 isolates were identified as *Enterecoccus* sp. and 22 of them were identified as *Lactococcus sp*. The rest of the 25 isolates which were morphologically identified as bacilli were evaluated as

Lactobacillus sp., they were capable to grow at a temperature of 15°C. Seventeen (17) of 22 isolates which were identified as *Lactococcus sp*. was estimated as *Lactococcus lactis* ssp*. lactis* (*L. lactis*), and three of them was estimated as *L. lactis* ssp*. cremoris* (*Lactococcus cremoris*), and two of them was estimated as *L. lactis* ssp*. lactis biovar. diacetylactis* (*Lactococcus diacetylactis*). In contrast to expected, five of these isolates that was identified as *L. lactis* could be grown in pH 9. 6 medium conditions, the other five could be grown at 45°C temperature, and seven of them could also be grown in medium which had 6.5% NaCl additive. Similar atypical reactions, in which strains were growing in various conditions such as pH 9.6, 45°C and 6.5% NaCl, were also observed for *Lactococcus sp.* isolates in various studies such as that of Tunail et al. (2001) who worked on Feta cheese, Bulut (2003)'s work on Pottery cheese and Kırmacı (2010)'s work on Urfa cheese. Unexpected positive and negative reactions also occurred in the tests where *Lactococcus* sp*.* was used to distinguish as subspecies. The results were evaluated as the subspecies-specific atypical reactions. It was noticed that the five isolates which were identified as *L. diacetylactis* and *L. lactis* could not generate ammoniac from arginine. In Garde et al. (1999) study, 13 of 57 arginine positive isolates isolated from cheeses produced from raw milk, were determined as *L. cremoris* and 44 of them were determine as *L. lactis*. Our study found out that, two isolates identified as *L. cremoris* could grow in a medium of 4% NaCl additive.

As a result of biochemical tests which provide identification based on physiological characteristics and nutrient requirements of bacteria, it is known that bacteria give atypical reactions because of adaptation to different conditions. Tolerance of bacteria isolated from cheeses with high salt concentration or whose clot is boiled, increasing with increase temperature or salt concentrations, respectively. Hence, it is believed that these cultures can adapt to these environment. Due to the resistance of difficult circumstances of *Enterecoccus* sp*.,* 36 isolates which showed positive reaction particularly to conditions such as 6.5% NaCl, pH 9.6, and temperature of 10 to 45°C, were separated as *Enterecoccus* sp*.,* 27 of them were identified as *Enterococcus faecalis* (*E. faecalis*) due to the 0.04% telluride reduction. 12 of 27 *E. faecalis* isolates could produce CO_2 by using citrate. *Entrecoccus* sp. is used as a starter culture in foods due to their properties such as lipolytic and esterolytic activity, citrate utilization and synthesis of volatile aromatic compounds (Vuyst et al., 2002; Giraffa, 2003; Erginkaya et al., 2007). *Enterococcus faecium* and *E. faecalis* in particular, can contribute to provide flavor for cheese due to their ability of producing compounds such as acetaldehyde, ethanol, diacetyl and acetone (Franz et al., 2003; Giraffa, 2003; Erginkaya et al., 2007). Twenty-four (24) *Enterecoccus* sp*.* were identified as 14 *E. faecalis*, five *Enterococcus durans*, three *Enterococcus hirae*, two *E. faecium* by using Gram-positive ID kit. The 11 *E. faecalis* isolates were similarly identified through both biochemical and carbohydrate tests but three of them was evaluated as *E. hirae* (two isolates) and *E. durans* (one isolates) as a result of a biochemical (carbohydrate) test.

Biochemically, identified 25 lactobacilli strains were evaluated as 12 *Lactobacillus plantarum (Lb. plantarum)* and 13 *Lactobacillus* sp*.* as a result of sugar tests and 16 of them were evaluated as seven *Lb. plantarum,* 7 *Enterococcus curvatus* and two *Enterococcus fermentum* by using API 50 CHL kit. Seven isolates were identified as *L. plantarum* as a result of both biochemical and carbohydrate tests (API 50 CHL kit) (Sharpe, 1979; Balows et al., 1991; Durlu-Özkaya et al., 2001).

Results of FTIR spectroscopy analysis

Bacterial spectra were formed as a result of chemical bonds in bacteria which absorbed infrared rays. The study found that spectra of a total of 91 samples (83 bacterial isolates and eight reference cultures) were taken at the range of 4000 and 450 cm^{-1} wavelength and 4 cm^{-1} resolution. Each sample was scanned 64 times with two parallel. The most dominant absorbance peaks were observed at ~ 3400, ~ 1600, ~ 1300, ~ 1100, ~ 600 cm^{-1} wavelengths. These wavelengths are divided into five windows by Naumann et al. (1990): 1. P1, Fatty acid region I (3000 to 2800 cm^{-1}); 2. P2, Amide Region (1800 to 1500 cm^{-1}); 3. P3, Combined Region (1500 to 1200 cm^{-1}); 4. P4, Polysaccharide Region (1200 to 900 cm^{-1}); 5. P5, Correct Fingerprint Region (900 to 700 cm^{-1}) windows.

FTIR spectrum of one of the reference strain at range of 4000 to 450 cm^{-1} wavelengths are shown in Figure 1. Pegram (2007) determined the dominant peaks to consist of water (3500 cm^{-1}), amide bonds (1700 to 1500 cm^{-1}), fatty acids (1500 to 1400 cm^{-1}) and polysaccharides (1200 to 900 cm^{-1}) regions. The separation of the bacteria at species and subspecies level was performed considering the same peaks of parallel bacteria, and different peaks of different bacteria at different absorbance. The fingerprint spectra were observed to be different and it was determined that *Pediococcus pentosaceus*, *Pediococcus acidilacdici*, *L. lactis ssp. lactis*, *Lactobacillus casei 201*, *L. casei 202*, *Lactobacillus helveticus*, *Pediococcus fluorescens* B 52 and *P. fluorescens* AFT 29 bacteria gave different peaks at repeated spectra. Pearson correlation product moment coefficient was applied to samples at all the 4500 to 450 cm^{-1} regions, like the P1 (3000 to 2800 cm-1) fatty acids region, P2 (1800 to 1500 cm^{-1}) amide region, P3 (1500 to 1200 cm^{-1}) combined region, P4 (1200 to 900 cm^{-1}) polysaccharide region and P5 (900 to 700 cm^{-1}) fingerprint region in order to determine the extent of similarity between two parallel of isolates and reference

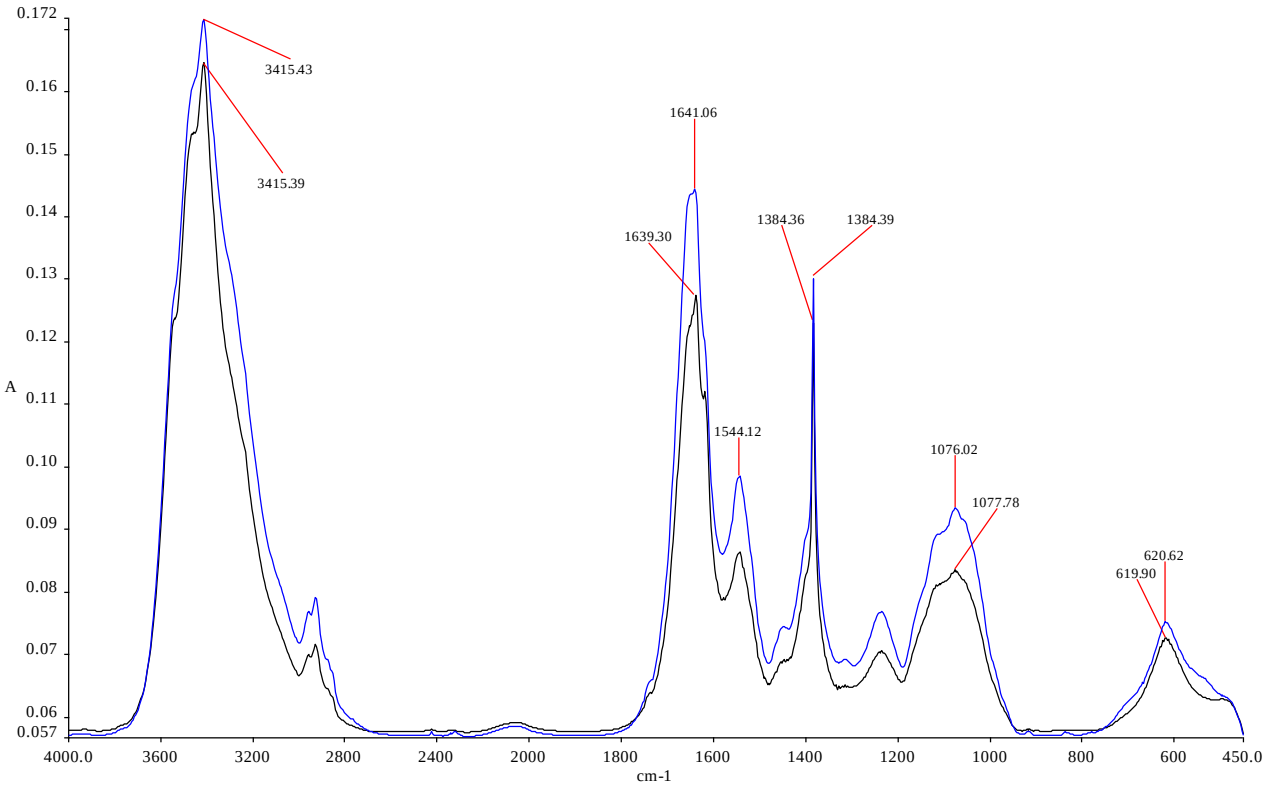

Figure 1. FTIR spectra of *L. lactis* at range of 4000 to 450 cm^{-1} wavelengths.

Table 1. Pearson correlations between the parallels of reference isolates at different wavelengths (Dziuba et al., 2007).

Reference strains	4000-450 cm^{-1} (%)	3000-2800 cm^{-1} (%)	1800-1500 cm^{-1} (%)	1500-1200 cm^{-1} (%)	1200-900 cm^{-1} (%)	900-700 cm^{-1} (%)
L. lactis	99.2	99.6	99.2	99.6	99.4	98.8
L. cremoris	99	99.4	83.2	99	96.9	35.3
L. diacetylactis	99.7	100	94	99.8	99.7	64
E. faecalis	97.6	99.7	99.2	97.4	100	99.7
E. faecium	99.1	99.5	94.5	98.4	98.6	94.5
Lb. plantarum	99.3	99.9	98.8	98.4	99.9	100
Lb. brevis	100	99.9	98.2	99.9	98.8	81.9
Str. thermophilus	96.2	98.2	94.8	96.1	95.7	91.1

strains samples (Table 1). Naumann et al. (1990) determined that the highest correlation was reached at the experiment with recurrence of reference strains at the following ranges; 3000 to 2800, 1500 to 1400 and 900 to 700 cm^{-1}. According to this study, correlation of the reference strains were found such as *L. lactis ssp. lactis* 99.9%, *L. lactis ssp. cremoris* 99.9%, *L. lactis ssp. lactis biovar. diacetylactis* 100%, *E. faecalis* 99.7%, *E. faecium* 99.9%, *Lactobacillus brevis* 99.9%, *L. plantarum* 99.9% and *Streptococcus thermophilus* 99.5%.

Similarity percentage of each isolates and each reference strains between their parallels were determined at the specified absorbance intervals (3000 to 2800, 1500 to 1400 and 900 to 700 cm^{-1}). Isolates and reference strains which have appropriate similarity rate (> 99%) with their parallels were averaged. Then, similarity percentages of averaged isolates with the averaged reference strains were determined by using Pearson correlation product moment coefficient. Isolates codes, biochemical tests results, API test results and isolates which gave the highest correlation ratios with the reference strains are given in Tables 2, 3 and 4.

According to Pearson correlation product moment coefficient, hierarchical clustering analysis was applied

Table 2. Comparison of the test results of *Lactococcus* sp.

Isolate	Biochemical tests results	Gram positive ID kit test results	API (%)	Pearson similarity (1. / 2. correlation)	r_{y1y2} (%)
İLc1	*L. lactis*	*L. lactis*	99.97	-	-
İLc2	*L. diacetylactis*	*L. lactis*	99.99	*E. faecalis* *L. lactis*	99.8 99.7
İLc3	*L.. cremoris*	-	-	*L. cremoris* *L. diacetylactis*	99.8 99.7
İLc4	*L. cremoris*	-	-	*E. faecalis* *L. lactis*	99.8 99.7
İLc5	*L. lactis*	*L. lactis*	99.99	-	-
iLc6	*L. lactis*	*L. lactis*	94.66	*L. cremoris* *L. diacetylactis*	99.7 99.6
İLc7	*L. lactis*	*L. lactis*	99.82	*L. cremoris* *L. diacetylactis*	99.7 99.7
İLc8	*L. lactis*	*L. cremoris*	94.66	*L. cremoris* *L. diacetylactis*	99.7 99.7
İLc9	*L. lactis*	*L. lactis*	99.49	*E. faecalis*	72.4
İLc10	*L. lactis*	*L. lactis*	99.95	*L. cremoris* *E. faecalis*	99.6 99.6
İLc11	*L. cremoris*	*Leuconostoc mesenteroides*	-	*L. lactis* *E. faecalis*	99.9 99.9
İLc12	*L. lactis*	*L. lactis*	99.99	*L. diacetylactis* *L. cremoris*	100 99.9
İLc13	*L. lactis*	*L. cremoris*	99.42	*L. diacetylactis, Str. thermophilus ve E. faecium*	99.9
İLc14	*L. lactis*	-	-	*E. faecalis* *L. lactis*	99.4 99.3
İLc15	*L. lactis*	*L. lactis*	91,21	*E. faecalis* *L. lactis*	97.2 96.7
İLc16	*L. lactis*	*L. lactis*	91.21	*L. lactis* *Str. thermophilus*	100
İLc17	*L. lactis*	-	-	-	
İLc18	*L. lactis*	*L. lactis*	99.99	*L. lactis* *E. faecalis*	95.6 95.6
İLc19	*L. lactis*	-	-	-	
İLc20	*L. diacetylactis*	-	-	*L. lactis* *Str. thermophilus ve E. faecalis*	100 99.9

Table 2. Contd.

İLc21	*L. lactis*	*L. lactis*	99.99	*E. faecalis* *L. lactis*	99.9 99.7
İLc22	*L. lactis*	*L. lactis*	99.99	*E. faecium* *L. diacetylactis,* *Str. thermophilus* *ve L. cremoris*	100 99.9

Table 3. Comparison of the tests results of *Enterecoccus* sp.

Isolate	Biochemical tests results	Gram positive ID kit test results	API (%)	Pearson similarity (1. / 2. correlation)	r_{y1y2} (%)
İE23	*Enterecoccus sp.*	-	-	*E. faecalis*	99.6
İE24	*Enterecoccus sp.*	*E. durans*	99.95	*E. faecium* *L. cremoris*	99.8
İE25	*E. faecalis*	*E. faecalis*	99.99	*L. diacetylactis* *L. cremoris*	99.8
İE26	*Enterecoccus sp.*	*E. durans*	99.99	*L. cremoris* *E. faecium* *L. diacetylactis*	99.8 99.7
İE27	*Enterecoccus sp.*	*E. durans*	99.99	*L. cremoris* *E. faecium* *L. diacetylactis*	99.7 99.6
İE28	*E. faecalis*	*E. faecalis*	99.99	*E. faecalis*	99.6
İE29	*Enterecoccus sp.*	*E. durans*	99.99	*E. faecalis* *L. lactis*	99.6 99.2
İE30	*E. faecalis*	-	-	*L. cremoris* *E. faecium* *L. diacetylactis*	100
İE31	*E. faecalis*	*E. faecalis*	99.87	*E. faecalis* *L. lactis*	99.7 99.7
İE32	*E. faecalis*	-	-	*E. faecalis* *L. lactis*	98.8 98.6
İE33	*Enterecoccus sp.*	-	-	*L. lactis* *Str. thermophilus*	100 99.9
İE34	*E. faecalis*	-	-	*L. lactis* *Str. thermophilus*	100
İE35	*E. faecalis*	*E. faecalis*	99.99	*Str. thermophiles* *L. lactis* *E. faecalis*	100 99.9
İE36	*E. faecalis*	*E. faecalis*	99.67	*Str. thermophiles* *L. lactis* *E. faecium*	100

Table 3. Contd.

İE37	*E. faecalis*	-	-	*E. faecalis* *L. lactis*	99
İE38	*E. faecalis*	-	-	*L. cremoris* *E. faecium* *L. diacetylactis*	100
İE39	*E. faecalis*	*E. faecalis*	99.67	*E. faecalis*	90
İE40	*E. faecalis*	*E. faecalis*	99.67	*E. faecalis* *L. lactis*	90.7 90.5
İE41	*E. faecalis*	-	-	*E. faecalis* *L. lactis*	99.9 99.8
İE42	*E. faecalis*	-	-	-	-
İE43	*E. faecalis*	*E. faecalis*	78.72	*E. faecalis* *L. lactis*	99.7 99.4
İE44	*E. faecalis*	*E. faecalis*	97.87	*L. cremoris, L. diacetylactis* *E. faecium* *Str. thermophilus*	100
İE45	*E. faecalis*	*E. faecalis*	99.97	*L. cremoris,* *E. faecium Str. thermophilus*	100
İE46	*E. faecalis*	*E. faecalis*	99.97	*E. faecalis*	99.8
İE47	*E. faecalis*	*E. faecalis*	99.97	*E. faecium Str. thermophilus*	100
İE48	*E. faecalis*	-	-	*L. diacetylactis*	100
İE49	*E. faecalis*	-	-	*L. diacetylactis L. cremoris*	100
İE50	*E. faecalis*	-	-	*L. diacetylactis*	100
İE51	*E. faecalis*	*E. hirae*	99.27	*L. lactis*	100
İE52	*E. faecalis*	*E. hirae*	98.88	-	-
İE53	*E. faecalis*	*E. durans*	99.95	*L. cremoris, L. diacetylactis* *E. faecium*	100
İE54	*E. faecalis*	*E. faecalis*	99.86	*E. faecalis* *L. lactis*	99.7 99.2
İE55	*E. faecalis*	*E. faecalis*	98.32	*L. cremoris,* *E. faecium*	99.8
İE56	*Enterecoccus sp.*	*E. faecalis*	99.96	*L. cremoris, L. diacetylactis* *E. faecium*	100

Table 3. Contd.

İE57	*Enterecoccus sp.*	*E. hirae*	98.59	*L. cremoris, L. diacetylactis* *E. faecium*	100
İE58	*Enterecoccus sp.*	*E. faecalis*	99.97	*L. cremoris,* *E. faecium*	99.7

Table 4. Comparison of the tests results of *Lactobacillus sp.*

Isolate	Biochemical tests results	Gram positive ID kit test results	API (%)	Pearson similarity (1. / 2. correlation)	r_{y1y2} (%)
İLb59	*Lactobacillus* sp.	*Lb. curvatus*	93.9	*Lb. plantarum* *Lb. brevis*	99.7 99.5
İLb60	*Lactobacillus* sp.	*Lb. curvatus*	99.9	*Lb. brevis* *Lb. plantarum*	99.9 99
İLb61	*Lactobacillus* sp.	-	-	*Lb. brevis* *Lb. plantarum*	100 98.9
İLb62	*Lb. plantarum*	*Lb. plantarum*	93.9	*Lb. brevis* *Lb. plantarum*	99.9 99.4
İLb63	*Lactobacillus sp.*	-	-	*Lb. brevis* *Lb. plantarum*	99.8 99.6
İLb64	*Lb. plantarum*	*Lb. plantarum*	93.9	-	-
İLb65	*Lb. plantarum*	*Lb. plantarum*	93.9	-	-
İLb66	*Lb. plantarum*	-	-	*Lb. brevis* *Lb. plantarum*	99.8 99.6
İLb67	*Lactobacillus* sp.	-	-	*Lb. brevis* *Lb. plantarum*	100 98.9
İLb68	*Lb. plantarum*	*Lb. plantarum*	93.9	*Lb. brevis* *Lb. plantarum*	99.9 99.3
İLb69	*Lactobacillus* sp.	-	-	*Lb. brevis* *Lb. plantarum*	99.9 99.5
İLb70	*Lb. plantarum*	-	-	*Lb. brevis* *Lb. plantarum*	99.9 99.4
İLb71	*Lb. plantarum*	-	-	*Lb. brevis* *Lb. plantarum*	100 99.1
İLb72	*Lb. plantarum*	-	-	*Lb. brevis* *Lb. plantarum*	99.9 99.2
İLb73	*Lb. plantarum*	-	-	*Lb. plantarum* *Lb. brevis*	99.8 99.5
İLb74	*Lactobacillus* sp.	*Lb. fermentum*	98.10	*Lb. brevis* *Lb. plantarum*	99.9 99.6

Table 4. Contd.

İLb75	*Lb. plantarum*	*Lb. plantarum*	93.9	*Lb. brevis* *Lb. plantarum*	100 99.2
İLb76	*Lactobacillus* sp.	*Lb. curvatus*	99.9	*Lb. plantarum* *Lb. brevis*	100 98.9
İLb77	*Lactobacillus* sp.	*Lb. fermentum*	98.10	*Lb. brevis* *Lb. plantarum*	99.9 99.2
İLb78	*Lb. plantarum*	*Lb. plantarum*	93.9	*Lb. brevis* *Lb. plantarum*	99.9 99.5
İLb79	*Lb. plantarum*	*Lb. plantarum*	93.9	*Lb. plantarum* *Lb. brevis*	96.8 92.7
İLb80	*Lactobacillus* sp.	*Lb. curvatus*	99.8	*Lb. brevis* *Lb. plantarum*	99.6 99.3
İLb81	*Lactobacillus* sp.	*Lb. curvatus*	99.8	*Lb. brevis* *Lb. plantarum*	99.8 98.9
İLb82	*Lactobacillus* sp.	*Lb. curvatus*	9.8	*Lb. brevis* *Lb. plantarum*	99.6 99
İLb83	*Lactobacillus* sp.	*Lb. curvatus*	99.8	*Lb. brevis* *Lb. plantarum*	99.8 99

to cultures whose similarity conditions were analyzed and isolates were divided into clusters by using the Ward method. Isolates evaluated as *Lactococcus sp.* were divided into two different clusters and while all of the reference strains were gathered in one cluster, 4 isolates (İLc4, İLc10, ILC3 and İLc2) formed a different cluster (Figure 2). Isolates considered to be *Enterecoccus* sp. were divided into four different clusters in two main clusters (Figure 3). While all of the reference strains were gathered in one main cluster, 7 isolates were collected in the other main cluster. Isolates evaluated as *Lactobacillus sp.* were divided two different clusters (Figure 4). While all of the reference strains were gathered in one cluster, 4 isolates (İLb80, İLb81, İLb82 and İLb83) formed a different cluster. Distances between isolates found in a different cluster outside the main cluster where the reference strains were found, were also considered. It was believed that those isolates might be originating from a different variety of isolates outside the known references.

Naumann et al. (1990) could successfully cluster *Staphylococcus*, *Streptococcus* and *Clostridium* at species level, at the ranges of 3000 to 2800, 1200 to 900, 900 to 700 cm^{-1} by applying cluster analysis and Pearson Pearson correlation product moment coefficient. Naumann et al. (1990) also applied cluster analysis to Clostridium type bacteria at the range of 1200 to 900 and 1500 to 1200 cm^{-1} region. In our study, appropriate fingerprint regions of reference strains could not be used to determine the statistical analysis due to inadequate reference strains been difficult to separate species to subspecies due to similar genotypic structure of LAB species. In order to identify distinctive fingerprint regions of LAB species using FTIR spectroscopy, it is thought that particular species are needed to compare with more than one reference strains according to different studies. For this reason, identification at species level using FTIR spectroscopy was not successfully carried out.

Conclusion

As a result, differences between the biochemical and the carbohydrate test results revealed the importance of the genotypic characterization at LAB's characterization. Twelve of 22 *Lactococcus* isolates were identified as *L. lactis* with biochemical and carbohydrate tests. However, this number was reduced to 6 following FTIR analysis. While 14 out of 36 *Enterecoccus* isolates were identified as *E. faecalis*, only 10 isolates were identified with FTIR analysis. Also, while 12 out of 25 *Lactobacillus* isolates were identified as *L. plantarum*, only four isolates were identified with FTIR analysis. 15.66% of total isolates were identified as *L. lactis*, 16.87% of total isolates were identified as *E. faecalis* and 8.43% of total isolates were identified as *L. plantarum*. It has been determined that in

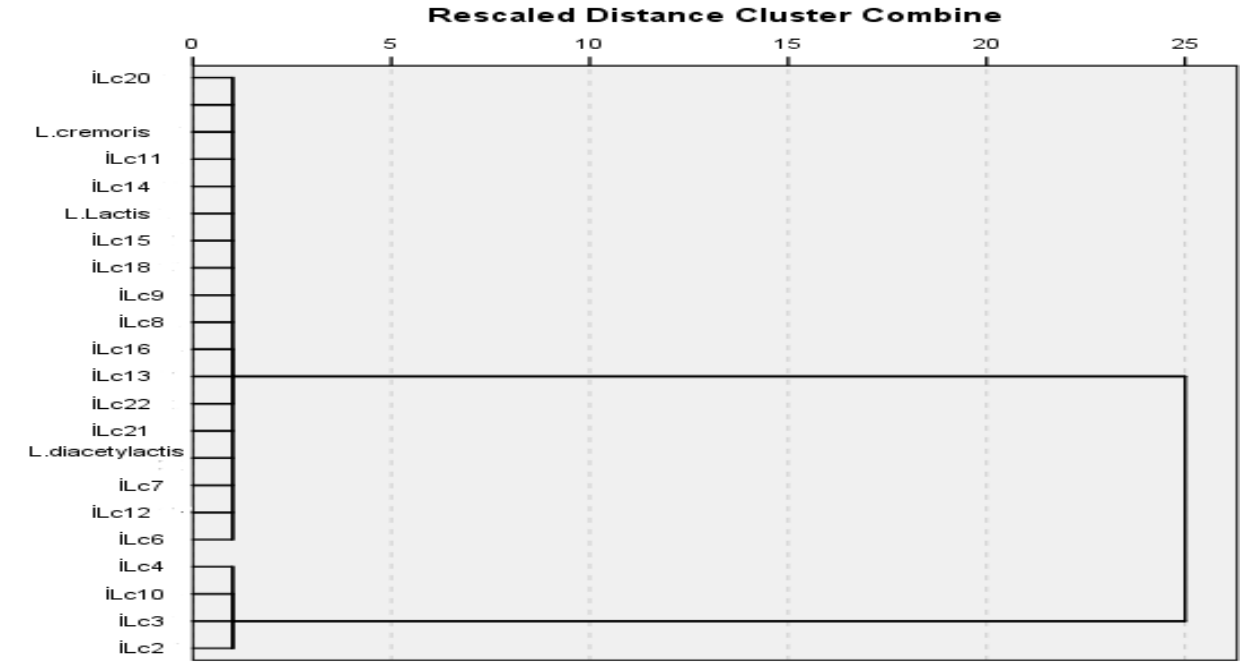

Figure 2. Dendogram of *Lactococcus sp.* by Ward's method.

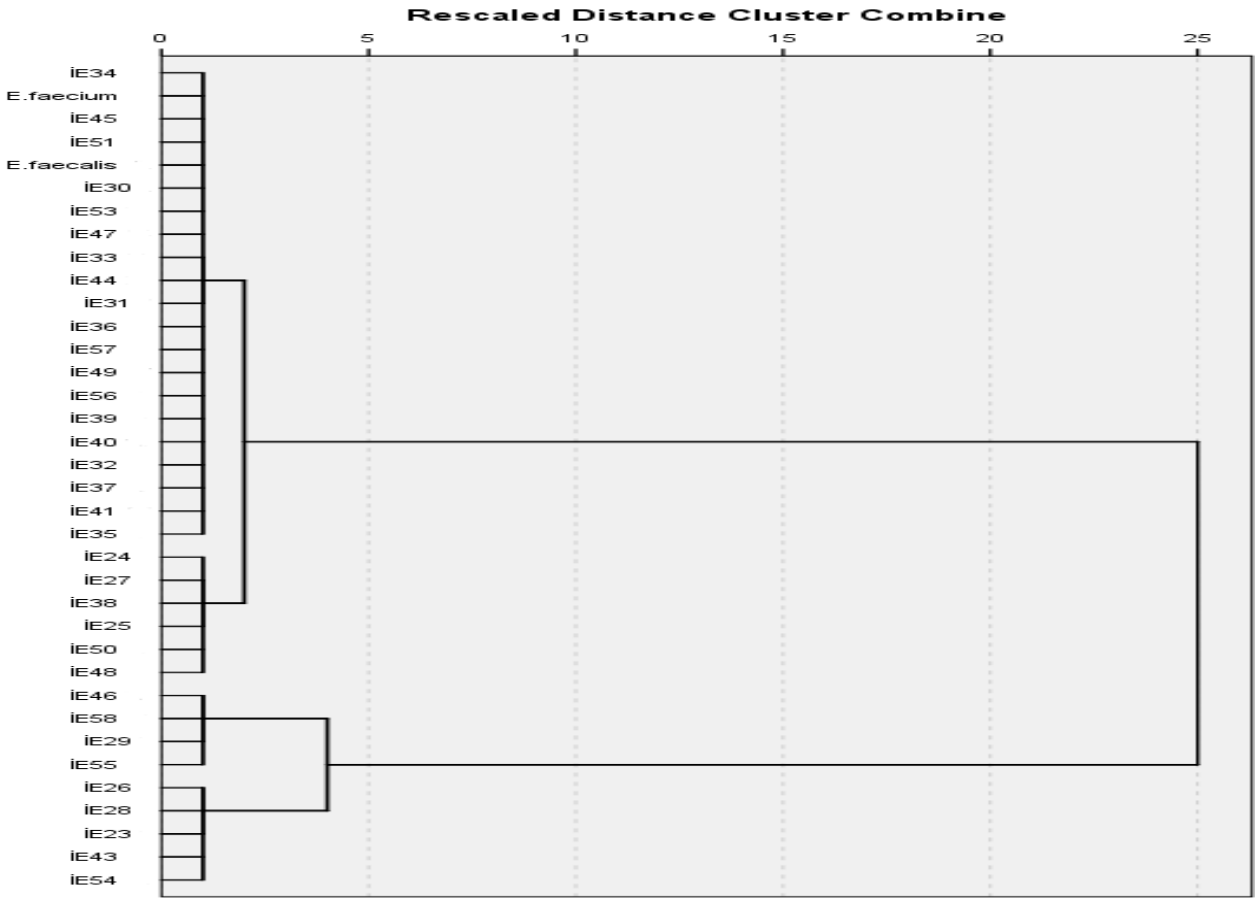

Figure 3. Dendogram of *Enterecoccus* sp. by Ward's method.

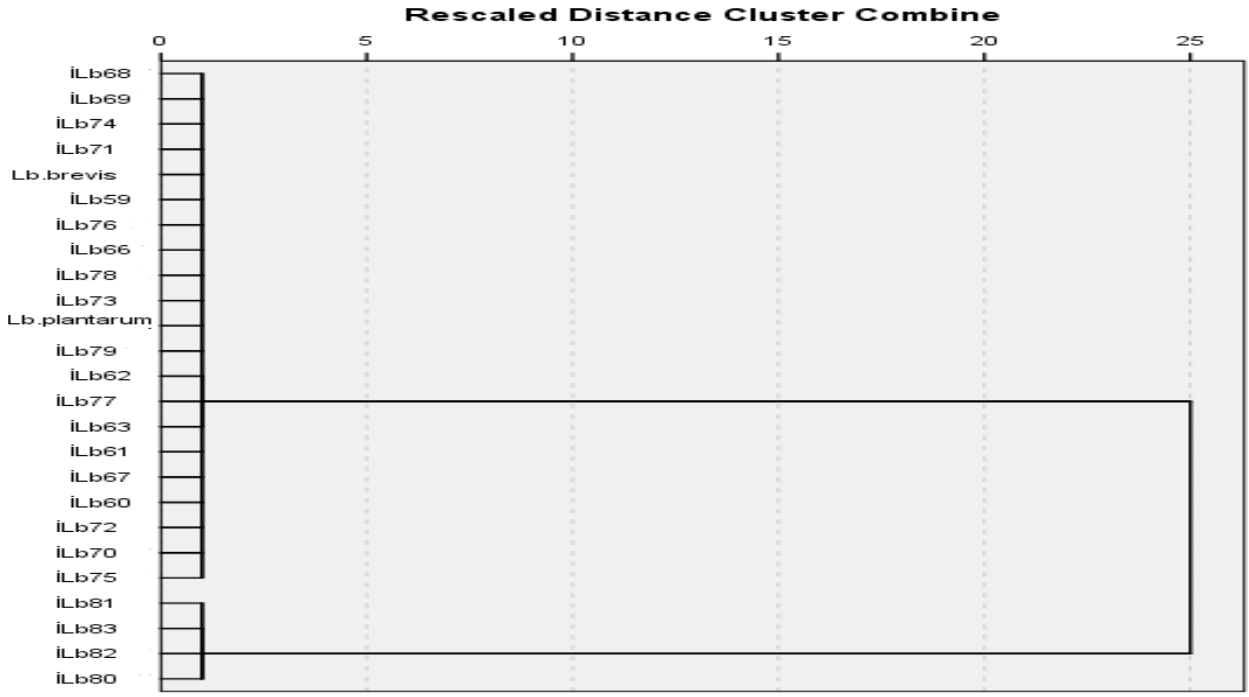

Figure 4. Dendogram of *Lactobacillus sp.* by Ward's method.

order to succeed in the identification of LAB with FTIR spectroscopy, a number of reference strains should be increased.

Conflict of interests

The authors did not declare any conflict of interest.

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Profile, antioxidant potential, and applicability of phenolic compounds extracted from *Spirulina platensis*

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This work aimed at an investigation of the profile of free phenolic compounds (PC) of *Spirulina platensis* and assesses their antioxidant potential, applying them as a natural conservative in minimally processed apples. The phenolic extract showed 396 µg g^{-1} gallic acid, 347 µg g^{-1} of caffeic acid, 54 µg g^{-1} salicylic acid and 3.5 µg g^{-1} trans-1-cinmamic a total of 608 µg PC g^{-1} of *S. platensis*. With the use of PC, it was possible to inhibit the radical DPPH over 180 min with IC_{50} of PC 202 µg mL^{-1}. The inhibition of polyphenol oxidase and peroxidase of PC were 19.9 and 9.7%. In addition, verifying the constants K_m and V_{max}, it was concluded that inhibition of the peroxidase and polyphenol occurs in an uncompetitive manner. Application of crude extract of PC under minimally processed apples showed inhibition of browning by 40%. The general acceptance of apples was not affected by the addition of PC.

Key words: Apple, enzymatic browning, peroxidase, phenols, polyphenol oxidase.

INTRODUCTION

The use of antioxidants in foods prevents the formation or propagation of free radicals resulting from the oxidation of various metabolic and environmental oxidative processes, thus reducing enzymatic browning and rancidity, which are the main oxidative processes occurring in these matrixes (Musa et al. 2013; Mai and Glomb, 2013). However, synthetic antioxidants are associated with a number of acute and chronic problems in humans (Hua-Bin et al., 2007), which has motivated the search for natural compounds with similar effects for use in large-scale. In the search for natural products with antioxidant activity, the microalgae belonging to the genus *Spirulina* stands out, which is commercialized and studied for its nutritional potential attributed to its protein content, pro-vitamins, unsaturated fatty acids and phenolic com-pounds (Derner et al., 2006). The use of bioproducts derived from these microalgae is favored by the viability of its large-scale cultivation and by the facility to optimize

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Abbreviations: PC, Phenolic compounds; **PPO,** polyphenol oxidase; **POD,** peroxidase.

the production of compounds of interest (Kepekçi et al., 2013).

Phenolic compounds are substances whose effectiveness as antioxidant agents is due to the ease of hydrogen atom donation to a free radical molecule (Giada and Mancini, 2006). The evaluation of antioxidant activity can be performed by various methods, always based on preventing or propagating the oxidation process. The most common is the DPPH free radical scavenging method (Noipa et al., 2011; Mishra et al., 2012). From the point of the food industry, preventing the oxidation process is a challenge to the safety of the product, especially concerning the new marketing methods, including the minimally processed products, in which the conventional heat treatment is not used for preserving the nutritional, functional and sensory characteristics of the product. In the case of plant products, the enzymes polyphenol oxidase and peroxidase are primarily responsible for the oxidative damage in fruits and vegetables, and their activation occurs when the tissue cells are ruptured during processing, while inactivation is done by the combination of heat treatment and conservatives in conventional products (Olivas et al., 2007). Because of the new food demands, such as minimally processed products, there is a need to seek innovative and effective ways to control oxidative enzymes in fruits. Given the benefits of phenolic compounds, they can be a promising alternative, but knowing its profile and its mechanisms of action is fundamental to recommend the use of these compounds.

This study aimed to investigate the profile of the free phenolic compounds from *Spirulina platensis*, and evaluate its antioxidant potential in the extract, aiming to apply them as conservative in minimally processed apples.

MATERIALS AND METHODS

Materials

S. platensis strain LEB-18 was isolated from Mangueira Lagoon (33° 302' 133" S; 53° 082' 593" W) and cultivated in a pilot plant located on the coast of this pond. Three tanks with surface area of 37.1 m^2 were lined with glass fiber, and covered by a greenhouse structure constructed from transparent polyethylene film. Cultures of *S. platensis* were stirred by rotating paddles at 18 rpm for 24 h during 387 days under natural light (Morais et al., 2009). The biomass was removed from the tanks, dried in an oven, ground in a Wiley mill, and stored in plastic containers under refrigeration until the extraction of phenolic compounds. The *S. platensis* LEB-18 has in its composition approximately 86% protein, 6.7% moisture and 3% lipids (Figueira et al., 2011). Apples (*Pirus malus*) varieties "Gala" were bought in a local market in the city of Rio Grande/RS, and kept under refrigeration (≈ 8°C). The reagents DPPH (95% purity), guaiacol, and the chromatographic patterns (98 to 99% purity) of gallic acid, trans-cinnamic acid, caffeic acid and salicylic acid were purchased from Sigma Chemicals (St. Louis, MO). Phenolic acids were dissolved in methanol from the stock solution, resulting in solutions of 100, 300 and 500 µg mL^{-1} for both gallic and trans-cinnamic acids, caffeic acid, and salicylic acid, respectively.

Determination of the free phenolic compounds (PC)

PC were extracted by adding 10 mL methanol to 3 g *S. platensis*, followed by horizontal shaking at 800 rpm for 1 h. The agitation was stopped for 15 min, after which 10 mL methanol was added and agitation was performed for 1 h. The methanol extract was filtered and evaporated on a rotary evaporator, and the residue was resuspended in distilled water and clarified with addition of 0.1 mol L^{-1} $Ba(OH)_2$ and 5% $ZnSO_4$ and allowed to rest for 20 min. The clarified extract was centrifuged, filtered, and the volume made up to 25 mL with distilled water (Souza et al., 2010). The PC quantification was carried out by mixing 1 mL phenolic extract, 4.5 mL alkaline solution (Na_2CO_3 4%: $CuSO_4$ 2%: $KNaC_4H_4O_6$ 4% at a ratio of 100:1:1, and allowed to rest for 15 min at 40°C. Subsequently, 0.5 mL Folin-Ciocalteau reagent (1:2, v/v) was added, and allowed to stand for 10 min. The absorbance was measured at 750 nm and the concentrations were calculated by a standard curve of gallic acid (from 2 to 30 mg mL^{-1}).

Phenolic acids profile

The determination of phenolic acids in the *S. platensis* extract was performed by HPLC-UV, with LC-AT pump connected to the degasser DGU with an integrator CBM-20A, 7725i manual injector, UV-VIS detector 10AXL, and software Shimadzu LC-Solutions. Separation was performed on a reversed phase column C18 CLC-ODS (150 mm × 4.6 mm ID 5 µm). A sample volume of 20 µL was injected into the column and eluted with a constant flow rate of 1.0 mL min^{-1}. Gradient elution was applied with "A" (water containing 1% acetic acid), "B" (methanol) and "C" (acetonitrile) as solvents, as follows: 0 min 31.5% B and 3.5% C; 3 min 38.7% B and 4.3% C; 10 min 53.1% B and 5.9% C; 12 min 31.5% B and 3.5% C, and total run time of 18 min. Each standard was assessed individually to check the retention times, confirmed by injection of three increasing concentrations to identify the compounds in the mixture. The linearity of the response was assessed using standard curves, with injections of five different concentrations, ranging from 2 to 37 µg mL^{-1} for gallic acid, from 0.4 to 7 µg mL^{1} for trans–cinnamic acid, from 11 to 176 µg mL^{-1} for caffeic acid and from 18 to 293 µg mL^{-1} for salicylic acid. The coefficients of the standard curves were obtained with the aid of software.

Antioxidant potential

The antioxidant potential of PC extracted from *S. platensis* was evaluated by spectrophotometry according to the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging method. The reaction was carried out with 3 mL DPPH solution ($5x10^{-5}$ mol L^{-1}), 1 mL phenolic extract (40 µg PC mL^{-1}) and 2 mL methanol. The mixture was stirred vigorously and allowed to stand for 30, 60, 90, 120, 150 and 180 min in the dark at room temperature (25°C). The consumption of the radical was evaluated at 515 nm. For the control test (time zero), the absorbance was measured immediately after mixing. Specific inhibition (SI) was calculated by the Equations 1 and 2. The inhibitory concentration (IC 50) was obtained by the concentration (µg mL^{-1}) of phenolic extract required to inhibit 50% of the initial DPPH radical.

$$\%I = [(Uabs1-Uabs2)/Uabs1] .100 \quad (1)$$

Where, Uabs 1 refers to the absorbance of the control, and Uabs 2 refers to the absorbance of the sample.

$$SI = \%I/PC \quad (2)$$

Where, I = inhibition; IE = Specific inhibition; PC = phenolic compounds.

Table 1. Wavelength (λ_{max}), retention time (t_R), analytical curve, and correlation coefficient (R) of standard phenolic acids.

Standard	t_R (min)	λ_{max} (nm)	Curve	R
Gallic acid	3.1	270	y= 97058.88 x - 129558.6	0.9987
Caffeic acid	4.0	323	y= 40102.72 x + 35500.59	0.9980
Salicylic acid	10.9	261	y= 4411.899 x - 8862.657	0.9996
Trans-cinnamic acid	14.9	277	y= 208924.5 x - 16602.11	0.9993

Potential inhibition of oxidative enzymes

Polyphenol oxidase (PPO) and peroxidase (POD) present in the crude enzyme extract from 'Gala' apples were used. The latter was obtained by stirring the apple pulp with 20 mmol L^{-1} phosphate buffer pH 6.0 (1:5, w/v) in a blender for 2 min. The homogenate was centrifuged at 3220 *g* for 10 min at 4°C, and then filtered and kept in this temperature (Oliveira et al., 2007). The enzymatic activity was determined by the reaction of 1 mL enzyme extract, 1.5 mL 20 mmol L^{-1} phosphate buffer pH 6.0, 2 mL distilled water, and 0.5 mL substrate specific for each enzyme, as follows: 0.1 N catechol for PPO and 1% guaiacol for POD. In the POD reaction, 1 mL 0.08% H_2O_2 was added. The reactions were carried out in a water bath at 30°C for 10 min. The protein of the extracts was determined by Lowry method (1951), in which a volume of 0.5 mL crude extract was mixed with 4 mL alkaline solution for 10 min. Then, 0.5 mL Folin reagent (1:2 v/v) was added and the mixture was allowed to stand for 30 min. A standard curve of albumin (from 0.05 to 0.42 mg mL^{-1}) was used for protein quantification. The readings were performed at 425, 470 and 660 nm for PPO, POD, and protein extracts reactions, respectively. The results for the specific enzyme activity were expressed as Uabs.mg $protein^{-1}$ where 1 Uabs represents an increase of 0.001 absorbance unit per minute. To identify the inhibition mechanism of the PC, the Michaelis-Mentem constant K_m and V_{max} was determined in the presence and absence of phenolic extracts. The enzymatic reactions were carried out by varying the concentrations of phenolic extract (4, 20 and 40 µg mL^{-1}) and the substrate (0 to 1.0 mL). In the control experiment, the phenolic extract was replaced by distilled water. The results were expressed in mg mL^{-1} for K_m and Uabs min^{-1} for $V_{máx}$.

Application of phenolic compounds in minimally processed apples

The apples were washed with detergent and sanitized with sodium hypochlorite (200 ppm/5 min). Then, the fruits were peeled, their seeds were removed, and the pulp was cut into pieces of 1.0 × 1.0 cm, and 4 cm length. Two treatments were performed with and without submersion in the PC extract. Apple samples from both treatments were packed in PVC film-wrapped expanded polystyrene trays, and kept under refrigeration (≈ 8°C) for 8 days. The pH of the minimally processed apples was measured in potentiometer Hanna model 200 (AOAC, 2000). The titratable acidity was determined according to AOAC (Kepekçi et al., 2013) and expressed as percentage of malic acid. The weight loss was determined by weighing the packages with the samples and the results were expressed as percentage (g 100 g^{-1}). The firmness of the samples was determined in a texture analyzer Stable Micro Systems (TA.XT Plus, England). The color of the samples was determined using a Minolta colorimeter (Chroma Meter CR400, Japan), by L, a*, b* system, and the browning (BI) index was calculated according to Fontes et al. (2008) as described in Equation 3:

$$BI = [100 (x-0.31)]/0.172 \quad (3)$$

Where, BI = browning index;

$$x = (a + 1.75 L)/[(5.645 L)+a (-3.02 b)$$

Statistical analysis

Statistical analysis was performed by analysis of variance (ANOVA) and Tukey's test for comparison between means, using the Software Statistics 5.0.

RESULTS AND DISCUSSION

Phenolic acid profile

S. platensis is considered an excellent source of phenolic acids including caffeic acid, chlorogenic acid, salicylic acid, synaptic acid, and trans-cinnamic acid (Colla et al., 2007). However, there are no studies on the profile of these phenolic acids extracted from *S. platensis* cultivated under the experimental conditions similar to the present study. Table 1 shows the retention times for the respective wavelengths, analytical curve and the correlation coefficient for each phenolic acid studied. The analytical curves of all phenolic acids had correlation values that allowed reliable quantification of the samples in the linearity range determined by the instrument (Ribani et al., 2004). The profile of *S. platensis* phenolic extract is shown in the chromatogram of Figure 1, which exhibits the separation of four compounds under the established conditions. The concentrations in µg g^{-1} (±CV) of the phenolic acids present in the *S. platensis* crude extract determined by HPLC-UV were 396±11.3, 347±7.6, 54±2.5, and 3.5±0.6 for galic acid, caffeic acid, salicylic acid, and trans-cinnamic acid, respectively, totaling 801 µg g^{-1} free phenolic acids. The PC concentration determined by spectrophotometry was 608 µg PC g^{-1} for *S. platensis*. This analysis was based on the reaction of the phenolic groups with Folin-Ciocalteau reagent, yielding a blue colored product, determined by spectrophotometry, whose value was 25% lower to that found by liquid chromatography (HPLC-UV), which enables the determination of each component without overlapping effects (Ribani et al., 2004).

Qualitative and quantitative differences in the same species can be due to the effect of abiotic and biotic variables, which are main determinant of the production of metabolic compounds with PC. In this context, Klejdus

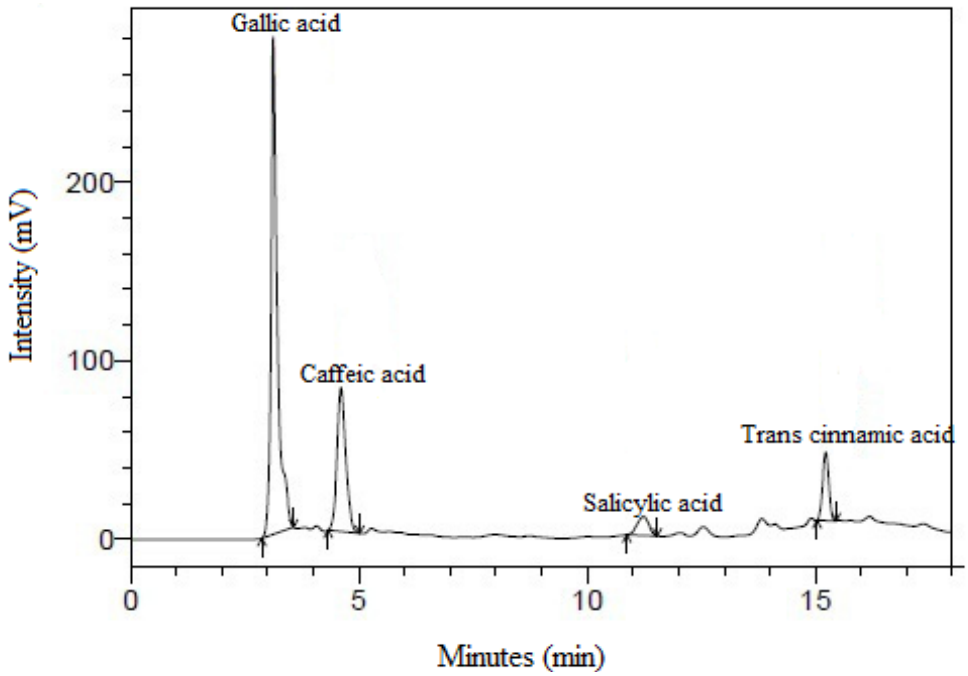

Figure 1. Chromatographic separation of phenolic acids by HPLC-UV.

et al. (2009) separated and identified phenols from microalgae by mass spectrometry, including *S. platensis*, which presented 0.169 µg g^{-1} caffeic acid, 0.072 µg g^{-1} chlorogenic acid, 0.254 µg g^{-1} vanillic acid and 2.23 µg g^{-1} p-hydroxybenzoic acid, which are lower values than those found in this study. This difference may be a consequence of the abiotic conditions.

Antioxidant activity on DPPH

The method used to estimate the antioxidant activity of the *Spirulina* phenolic extract is based on the electron transfer from an antioxidant compound to a free radical, DPPH that, when it is reduced, loses its purple color (Duarte-Almeida et al., 2006). The procedure is fast and allows evaluating the ability to inhibit the propagation phase of the oxidation process. In the present study, the inhibition potential of the extract was studied over 180 min (Table 2) as specific inhibition (% inhibition μg^{-1} PC), demonstrating the ability to inhibit DPPH. This ability and stability are very promising to inhibit the enzymatic browning in the fruits. The IC_{50} of the PC estimated for the reduction of DPPH is 202 µg PC mL^{-1} phenolic extract, which is similar to that reported by Mendiola et al. (2007), who found 297 µg mL^{-1}, extracting PC from *S. platensis* by supercritical extraction. It should be emphasized that the lower the IC_{50} the larger the consumption of DPPH by a sample and the greater its antioxidant activity (Sousa et al., 2007; Morais et al., 2009).

Table 2. Specific inhibition of PC from *S. platensis* on DPPH.

Time (min)	Specific inhibition * (%CV**)
0	0.023[b] (12.5)
30	0.241[a] (6.1)
60	0.269[a] (7.5)
90	0.348[a] (4.9)
120	0.345[a] (8.0)
150	0.305[a] (4.5)
180	0.361[a] (5.2)

Same letters in the column do not differ statistically ($p < 0.05$); *% inhibition / µg PC; ** CV= coefficient of variation ($n = 3$).

Antioxidant activity on POD and PPO

The inhibitory effect of PC in catalyzed oxidation processes is still poorly investigated, once there are some phenolic compounds among the substrates of the oxidative enzymes. Thus, the enzyme activity in adverse situations results in oxidation and polymerization of derivatives to protect tissue from injury. However, some phenolic compounds may act as inhibitors of enzymatic browning (Oliveira et al., 2007). The POD and PPO from crude enzyme extract of 'Gala' apples were inhibited by 19.9 and 9.7% µg of PC mL^{-1}, respectively, in the presence of the phenolic acid extract from *S. platensis*. Colla et al. (2007) found inhibition values of 35% for POD

Table 3. K_m values (mg mL^{-1}) and $V_{máx}$ (Uabs min^{-1}) of the enzymatic browning in the presence and absence of *S. platensis* phenolic extract.

PC (µg mL^{-1})	Peroxidase		Polyphenoloxidase	
	K_m	$V_{máx}$	K_m	$V_{máx}$
Control	0.33	0.05	0.03	0.06
4	0.14	0.01	0.02	0.02
20	0.13	0.02	0.02	0.02
40	0.01	0.01	0.02	0.03

Table 4. Physicochemical characteristics of minimally processed apples, subjected to treatment with PC extracted from *S. platensis* and control.

Condition	Time (days)	Parameters				
		BI**	Firmness (N)	Weight loss (%)	Acidity*	pH
Control	0	68.27	5.01	0	0.2	4.3
	2	57.93	11.15	1.9	0.2	4.3
	4	58.39	4.56	3.6	0.2	4.4
	6	78.56	2.53	5.7	0.2	4.4
	8	70.18	5.95	7.4	0.2	4.3
With PC	0	48.39	14.18	0	0.2	4.3
	2	41.41	31.61	0.8	0.2	4.3
	4	46.70	24.39	4.4	0.2	4.3
	6	49.99	24.83	6.7	0.2	4.2
	8	52.18	26.04	8.8	0.1	4.2

*expressed in % malic acid g^{-1} sample; ** BI.

extracted from potato at a *S. platensis* concentration of phenolic compounds of 4.9 mg g^{-1} dry biomass. The enzyme extract used in the test was not purified to better verify the potential of the PC extract to protect the apples against enzymatic browning if it would be applied in processing them. The Michaelis-Mentem constant K_m and V_{max} for POD and PPO in the presence of the *Spirulina* extracts are shown in Table 3 (Schnell and Maini, 2003). A decrease in the maximum velocity ($V_{máx}$) and substrate consumption (K_m) was observed with the addition of PC for both studied enzymes. This reduction is a characteristic of uncompetitive inhibition, in which the inhibitor interacts with the enzyme-substrate complex, preventing formation of the ternary enzyme-substrate-inhibitor complex to release the product (dark pigments).

Thus, the POD or PPO possibly binds to their preferred substrates and subsequently to the PC, forming the complex. The greater the affinity between the enzyme and its substrate, the faster is its inhibition, that is the formation of a complex with the compound added to the medium to inhibit enzymatic browning.

Application of PC in minimally processed 'Gala' apples

The use of synthetic antioxidants in foods such as sodium bisulfite, butylated hydroxyanisole, butylated hydroxytoluene, among others, are restricted by their respective toxicities that depend on its concentration in the product, besides the negative effect on the texture, aroma and taste of the processed product (Teixeira and Monteiro, 2006; Machado and Toledo, 2006). *S. platensis* is considered a GRAS microorganism (generally accepted as safe) permitted as a food additive by the Food and Drug Administration (FDA). Thus, the PC extracted from these microalgae can be applied into minimally processed apples, with benefit of the possibility of the PC to act as antioxidant against the enzymatic browning, and possible synergistic effect on the product functionality. To illustrate the treatment effect with PC extracted from *S. platensis* on the characteristics of minimally processed apples, Table 4 shows the results for browning index, firmness, weight loss, titratable acidity and pH determined over 8 days of experiment. Although, the browning index increased over time for both samples, the apple samples treated with PC presented lower values than the untreated samples. The browning inhibition was on average 40% as compared to the control samples. The browning index during the experiment was variable because the enzymes that cause browning are unevenly distributed without homogeneity across the apple, since the whole pulp of

the apple samples were used in the experiment (Oliveira et al., 2007). The weight loss increased with storage time, being one of the characteristics that interfere with the acceptability of the product by the consumer as it affects the turgidity of the tissues (Chitarra and Chitarra, 2005). However, it may not be directly related to the enzymatic browning.

Organic acids in foods influence the acceptance of a particular product, modifying its sensory properties. For this reason, these compounds were determined, since they indicate the effect of other compounds altered during aging. The acidity values also varied from 0.1 to 0.2% malic acid, similar to the study carried out by Drake et al. (1993) with fruits of the variety *'Golden Delicious'* stored at different CO_2 levels (0.18 to 0.19% malic acid). The pH parameter has great importance on oxidative browning of plant tissues, since its decrease may result in reducing the rate of enzymatic browning of a fruit besides being an indicator of microbial activity (Carvalho et al., 2005). The pH values ranged from 4.3 to 4.2, and were higher than those values found by Fontes et al. (2008) for 'Gala' apples treated with a preservative solution (ascorbic acid, citric acid, calcium chloride, sodium-chloride) in the order of pH 3.5. With respect to firmness, the samples treated with PC presented values 76% higher values than the control samples. The firmness loss mainly occurs due to the action of proteolytic and pectinolytic enzymes on the cell wall components (Belloso et al., 2007).

In the present study, the PC may have also inhibited the action of these enzymes. The variability of the results during the experiment may be caused by the formation of ligno-suberized boundary layer in the cutting zone of the fruits (Empís and Moldão-Martins, 2000). It has been demonstrated that the PC from natural sources such as that extracted from *S. platensis* is very promising to replace the conventional conservatives, aimed to prevent browning catalyzed by the oxidative enzymes and to preserve sensory properties improving the consumer acceptance of the products.

Conclusion

The PC extracted from *S. platensis* exhibited antioxidant activity on both the synthetic DPPH radical and oxidative enzymes such as POD and PPO of apples. When applied to minimally processed apples, the PC was effective in reducing oxidative browning, besides preserving other acceptance characteristics of the product. The natural antioxidants tested could be an interesting alternative to minimize or delay the oxidative deterioration, allowing the extension of the induction period, thus contributing to strengthening the supply of functional compounds.

Conflict of interests

The authors did not declare any conflict of interest.

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