African Journal of Microbiology Research

Volume 10 Number 43 21 November, 2016

ISSN 1996-0808

ABOUT AJMR

The African Journal of Microbiology Research (AJMR) is published weekly (one volume per year) by Academic Journals.

The African Journal of Microbiology Research (AJMR) provides rapid publication (weekly) of articles in all areas of Microbiology such as: Environmental Microbiology, Clinical Microbiology, Immunology, Virology, Bacteriology, Phycology, Mycology and Parasitology, Protozoology, Microbial Ecology, Probiotics and Prebiotics, Molecular Microbiology, Biotechnology, Food Microbiology, Industrial Microbiology, Cell Physiology, Environmental Biotechnology, Genetics, Enzymology, Molecular and Cellular Biology, Plant Pathology, Entomology, Biomedical Sciences, Botany and Plant Sciences, Soil and Environmental Sciences, Zoology, Endocrinology, Toxicology. The Journal welcomes the submission of manuscripts that meet the general criteria of significance and scientific excellence. Papers will be published shortly after acceptance. All articles are peer-reviewed.

Contact Us

Editorial Office: ajmr@academicjournals.org

Help Desk: helpdesk@academicjournals.org

Website: http://www.academicjournals.org/journal/AJMR

Submit manuscript online http://ms.academicjournals.me/

Editors

Prof. Stefan Schmidt
Applied and Environmental Microbiology
School of Biochemistry, Genetics and Microbiology
University of KwaZulu-Natal
Pietermaritzburg,
South Africa.

Prof. Fukai Bao
Department of Microbiology and Immunology
Kunming Medical University
Kunming,
China.

Dr. Jianfeng Wu
Dept. of Environmental Health Sciences
School of Public Health
University of Michigan
USA.

Dr. Ahmet Yilmaz Coban
OMU Medical School
Department of Medical Microbiology
Samsun,
Turkey.

Dr. Seyed Davar Siadat
Pasteur Institute of Iran
Pasteur Square, Pasteur Avenue
Tehran,
Iran.

Dr. J. Stefan Rokem
The Hebrew University of Jerusalem
Department of Microbiology and Molecular Genetics
Jerusalem,
Israel.

Prof. Long-Liu Lin
National Chiayi University
Chiayi,
Taiwan.

Dr. Thaddeus Ezeji
Fermentation and Biotechnology Unit
Department of Animal Sciences
The Ohio State University
USA.

Dr. Mamadou Gueye
MIRCEN/Laboratoire commun de microbiologie
IRD-ISRA-UCAD
Dakar, Senegal.

Dr. Caroline Mary Knox
Department of Biochemistry, Microbiology and Biotechnology
Rhodes University
Grahamstown,
South Africa.

Dr. Hesham Elsayed Mostafa
Genetic Engineering and Biotechnology Research Institute (GEBRI)
Mubarak City For Scientific Research
Alexandria, Egypt.

Dr. Wael Abbas El-Naggar
Microbiology Department
Faculty of Pharmacy
Mansoura University
Mansoura, Egypt.

Dr. Barakat S.M. Mahmoud
Food Safety/Microbiology
Experimental Seafood Processing Laboratory
Costal Research and Extension Center
Mississippi State University
Pascagoula,
USA.

Prof. Mohamed Mahrous Amer
Faculty of Veterinary Medicine
Department of Poultry Diseases
Cairo university
Giza, Egypt.

Editors

Dr. R. Balaji Raja
Department of Biotechnology
School of Bioengineering
SRM University
Chennai,
India.

Dr. Aly E Abo-Amer
Division of Microbiology
Botany Department
Faculty of Science
Sohag University
Egypt.

Editorial Board Members

Dr. Haoyu Mao
Department of Molecular Genetics and Microbiology
College of Medicine
University of Florida
Florida, USA.

Dr. Yongxu Sun
Department of Medicinal Chemistry and
Biomacromolecules
Qiqihar Medical University
Heilongjiang
P.R. China.

Dr. Ramesh Chand Kasana
Institute of Himalayan Bioresource Technology
Palampur,
India.

Dr. Pagano Marcela Claudia
Department of Biology,
Federal University of Ceará - UFC
Brazil.

Dr. Pongsak Rattanachaikunsopon
Department of Biological Science
Faculty of Science
Ubon Ratchathani University
Thailand.

Dr. Gokul Shankar Sabesan
Microbiology Unit, Faculty of Medicine
AIMST University
Kedah,
Malaysia.

Editorial Board Members

Dr. Kamel Belhamel
Faculty of Technology
University of Bejaia
Algeria.

Dr. Sladjana Jevremovic
Institute for Biological Research
Belgrade,
Serbia.

Dr. Tamer Edirne
Dept. of Family Medicine
Univ. of Pamukkale
Turkey.

Dr. Mohd Fuat ABD Razak
Institute for Medical Research
Malaysia.

Dr. Minglei Wang
University of Illinois at Urbana-Champaign
USA.

Dr. Davide Pacifico
Istituto di Virologia Vegetale – CNR
Italy.

Prof. N. S. Alzoreky
Food Science & Nutrition Department
College of Agricultural Sciences & Food
King Faisal University
Saudi Arabia.

Dr. Chen Ding
College of Material Science and Engineering
Hunan University
China.

Dr. Sivakumar Swaminathan
Department of Agronomy
College of Agriculture and Life Sciences
Iowa State University
USA.

Dr. Alfredo J. Anceno
School of Environment, Resources and Development (SERD)
Asian Institute of Technology
Thailand.

Dr. Iqbal Ahmad
Aligarh Muslim University
Aligrah,
India.

Editorial Board Members

Dr. Juliane Elisa Welke
UFRGS – Universidade Federal do Rio Grande do Sul
Brazil.

Dr. Iheanyi Omezuruike Okonko
Department of Virology
Faculty of Basic Medical Sciences
University of Ibadan
Ibadan,
Nigeria.

Dr. Giuliana Noratto
Texas A&M University
USA.

Dr. Babak Mostafazadeh
Shaheed Beheshty University of Medical Sciences
Iran.

Dr. Mehdi Azami
Parasitology & Mycology Department
Baghaeei Lab.
Isfahan,
Iran.

Dr. Rafel Socias
CITA de Aragón
Spain.

Dr. Anderson de Souza Sant'Ana
University of São Paulo
Brazil.

Dr. Juliane Elisa Welke
UFRGS – Universidade Federal do Rio Grande do Sul
Brazil.

Dr. Paul Shapshak
USF Health
Depts. Medicine and Psychiatry & Beh Med.
Div. Infect. Disease & Internat Med
USA.

Dr. Jorge Reinheimer
Universidad Nacional del Litoral (Santa Fe)
Argentina.

Dr. Qin Liu
East China University of Science and Technology
China.

Dr. Samuel K Ameyaw
Civista Medical Center
USA.

Dr. Xiao-Qing Hu
State Key Lab of Food Science and Technology
Jiangnan University
China.

Prof. Branislava Kocic
University of Nis
School of Medicine
Institute for Public Health
Nis,
Serbia.

Prof. Kamal I. Mohamed
State University of New York
Oswego,
USA.

Dr. Adriano Cruz
Faculty of Food Engineering-FEA
University of Campinas (UNICAMP)
Brazil.

Dr. Mike Agenbag
Municipal Health Services,
Joe Gqabi,
South Africa.

Dr. D. V. L. Sarada
Department of Biotechnology
SRM University
Chennai
India.

Prof. Huaizhi Wang
Institute of Hepatopancreatobiliary
Surgery of PLA Southwest Hospital
Third Military Medical University
Chongqing
China.

Prof. A. O. Bakhiet
College of Veterinary Medicine
Sudan University of Science and Technology
Sudan.

Dr. Saba F. Hussain
Community, Orthodontics and Peadiatric Dentistry Department
Faculty of Dentistry
Universiti Teknologi MARA
Selangor,
Malaysia.

Editorial Board Members

Prof. Zohair I. F. Rahemo
Department of Microbiology and Parasitology
Clinical Center of Serbia
Belgrade,
Serbia.

Dr. Afework Kassu
University of Gondar
Ethiopia.

Dr. How-Yee Lai
Taylor's University College
Malaysia.

Dr. Nidheesh Dadheech
MS. University of Baroda,
Vadodara,
India.

Dr. Franco Mutinelli
Istituto Zooprofilattico Sperimentale delle Venezie
Italy.

Dr. Chanpen Chanchao
Department of Biology,
Faculty of Science,
Chulalongkorn University
Thailand.

Dr. Tsuyoshi Kasama
Division of Rheumatology,
Showa University
Japan.

Dr. Kuender D. Yang
Chang Gung Memorial Hospital
Taiwan.

Dr. Liane Raluca Stan
University Politehnica of Bucharest
Department of Organic Chemistry
Romania.

Dr. Mohammad Feizabadi
Tehran University of Medical Sciences
Iran.

Prof. Ahmed H Mitwalli
Medical School
King Saud University
Riyadh,
Saudi Arabia.

Dr. Mazyar Yazdani
Department of Biology
University of Oslo
Blindern,
Norway.

Dr. Babak Khalili Hadad
Department of Biological Sciences
Islamic Azad University
Roudehen,
Iran.

Dr. Ehsan Sari
Department of Plant Pathology
Iranian Research Institute of Plant Protection
Tehran,
Iran.

Dr. Snjezana Zidovec Lepej
University Hospital for Infectious Diseases
Zagreb,
Croatia.

Dr. Dilshad Ahmad
King Saud University
Saudi Arabia.

Dr. Adriano Gomes da Cruz
University of Campinas (UNICAMP)
Brazil

Dr. Hsin-Mei Ku
Agronomy Dept.
NCHU
Taichung,Taiwan.

Dr. Fereshteh Naderi
Islamic Azad University
Iran.

Dr. Adibe Maxwell Ogochukwu
Department of Clinical Pharmacy and Pharmacy Management,
University of Nigeria
Nsukka,
Nigeria.

Dr. William M. Shafer
Emory University School of Medicine
USA.

Dr. Michelle Bull
CSIRO Food and Nutritional Sciences
Australia.

Editorial Board Members

Prof. Márcio Garcia Ribeiro
School of Veterinary Medicine and Animal Science-UNESP,
Dept. Veterinary Hygiene and Public Health,
State of Sao Paulo
Brazil.

Prof. Sheila Nathan
National University of Malaysia (UKM)
Malaysia.

Prof. Ebiamadon Andi Brisibe
University of Calabar,
Calabar,
Nigeria.

Dr. Julie Wang
Burnet Institute
Australia.

Dr. Jean-Marc Chobert
INRA- BIA, FIPL
France.

Dr. Zhilong Yang
Laboratory of Viral Diseases
National Institute of Allergy and Infectious Diseases,
National Institutes of Health
USA.

Dr. Dele Raheem
University of Helsinki
Finland.

Dr. Biljana Miljkovic-Selimovic
School of Medicine,
University in Nis,
Serbia.

Dr. Xinan Jiao
Yangzhou University
China.

Dr. Endang Sri Lestari, MD.
Department of Clinical Microbiology,
Medical Faculty,
Diponegoro University/Dr. Kariadi Teaching Hospital,
Semarang
Indonesia.

Dr. Hojin Shin
Pusan National University Hospital
South Korea.

Dr. Yi Wang
Center for Vector Biology
Rutgers University
New Brunswick
USA.

Prof. Natasha Potgieter
University of Venda
South Africa.

Dr. Sonia Arriaga
Instituto Potosino de Investigación Científicay Tecnológica/
División de Ciencias Ambientales
Mexico.

Dr. Armando Gonzalez-Sanchez
Universidad Autonoma Metropolitana Cuajimalpa
Mexico.

Dr. Pradeep Parihar
Lovely Professional University
Punjab,
India.

Dr. William H Roldán
Department of Medical Microbiology
Faculty of Medicine
Peru.

Dr. Kanzaki, L. I. B.
Laboratory of Bioprospection
University of Brasilia
Brazil.

Prof. Philippe Dorchies
National Veterinary School of Toulouse,
France.

Dr. C. Ganesh Kumar
Indian Institute of Chemical Technology,
Hyderabad
India.

Dr. Zainab Z. Ismail
Dept. of Environmental Engineering
University of Baghdad
Iraq.

Dr. Ary Fernandes Junior
Universidade Estadual Paulista (UNESP)
Brasil.

Editorial Board Members

Dr. Fangyou Yu
The first Affiliated Hospital of Wenzhou Medical College
China.

Dr. Galba Maria de Campos Takaki
Catholic University of Pernambuco
Brazil.

Dr Kwabena Ofori-Kwakye
Department of Pharmaceutics
Kwame Nkrumah University of Science & Technology
Kumasi,
Ghana.

Prof. Liesel Brenda Gende
Arthropods Laboratory,
School of Natural and Exact Sciences,
National University of Mar del Plata
Buenos Aires,
Argentina.

Dr. Hare Krishna
Central Institute for Arid Horticulture
Rajasthan,
India.

Dr. Sabiha Yusuf Essack
Department of Pharmaceutical Sciences
University of KwaZulu-Natal
South Africa.

Dr. Anna Mensuali
Life Science
Scuola Superiore Sant'Anna
Italy.

Dr. Ghada Sameh Hafez Hassan
Pharmaceutical Chemistry Department
Faculty of Pharmacy
Mansoura University
Egypt.

Dr. Kátia Flávia Fernandes
Department of Biochemistry and Molecular Biology
Universidade Federal de Goiás
Brasil.

Dr. Abdel-Hady El-Gilany
Department of Public Health & Community Medicine
Faculty of Medicine
Mansoura University
Egypt.

Dr. Radhika Gopal
Cell and Molecular Biology
The Scripps Research Institute
San Diego, CA
USA.

Dr. Mutukumira Tony
Institute of Food Nutrition and Human Health
Massey University
New Zealand.

Dr. Habip Gedik
Department of Infectious Diseases and Clinical Microbiology
Ministry of Health Bakırköy Sadi Konuk Training and Research Hospital
Istanbul,
Turkey.

Dr. Annalisa Serio
Faculty of Bioscience and Technology for Food Agriculture and Environment
University of Teramo
Teramo,
Italy.

African Journal of Microbiology Research

Table of Contents: Volume 10 Number 43 21 November, 2016

ARTICLES

Partial characterization of bacteriocin-like substance produced by probiotic *Lactobacillus plantarum* F12 isolated from Algerian children faeces **1798**
Fathia BAHRI and Noreddine KACEM CHAOUCHE

Effectiveness assessment of mouthwashes formulated from the essential oils of some beninese medicinal plants against oral germs **1806**
Jacob Bonou, Farid Baba-Moussa, Pacôme Agossou Noumavo, Adonice Zannou, Diane Aloukoutou, Lisette Kpavode, Hélène Ahouandjinou, Joachim Djimon Gbénou, Fatiou Toukourou and Lamine Baba-Moussa

Characterization of arbuscular mycorrhizal fungi in apple (*Malus domestica* Borkh) growing area in Kashmir Himalaya (India): A case study of Bandipora district **1813**
Misbah A. Malik, Zargar M. Y. and Baba Z. A.

Storage behaviour and functionality of a heat stabilized starter derived from an African opaque sorghum beer **1822**
Djegui K. Y., A P. P. Kayodé and J. D. Hounhouigan

Selection of anti-wetting agents and photoprotectants compatible with a soybean weed control bioagent **1829**
Machado, A. C. R., Monteiro, A. C. and Mochi, D. A.

Identification and tracking of microorganisms from the biofilms of container walls used for water storage: Case of rural communities in Burkina Faso **1837**
Seyram K. Sossou, Franck Lalanne, Mariam Dakoure/Sou and Naoyuki Funamizu

academicJournals

Vol. 10(43), pp. 1798-1805, 21 November, 2016
DOI: 10.5897/AJMR2016.8298
Article Number: 943FF0261657
ISSN 1996-0808
Copyright © 2016
Author(s) retain the copyright of this article
http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Full Length Research Paper

Partial characterization of bacteriocin-like substance produced by probiotic *Lactobacillus plantarum* F12 isolated from Algerian children faeces

Fathia BAHRI* and Noreddine KACEM CHAOUCHE

Laboratoire de Mycologie, de Biotechnologie et de l'Activité Microbienne, Université Mentouri, Constantine - Algeria.

Received 8 September, 2016: Accepted 19 October, 2016.

The strain, *Lactobacillus plantarum* F12 with probiotic traits was isolated from Algerian healthy children faeces and identified by 16S rDNA sequencing. In this study, the antimicrobial activity and physicochemical properties of bacteriocin-like substance (BLS) produced by this strain were determined. Also, the bacteriocinogenic genes of plantaricin A, plantaricin J and plantaricin K were screened in this strain. The BLS inhibited a range of pathogenic and spoilage bacteria including *Escherichia coli, Salmonella infantis, Salmonella typhimurium*, *Shigella sonnei, Staphylococcus aureus, Listeria monocytogenes, Listeria innocua, Bacillus subtilis* and some lactobacilli sp. The BLS was proteinaceous since it was inactivated by the proteolytic enzymes (trypsin, proteinase K and pronase) but not by α-amylase and lipase. It was heat stable at different temperatures (40 - 121°C) for 30 min and retained its activity at a wide range of pH values (2 to 10). Its activity was totally preserved at -80°C for 120 days and at -20°C for 60 days. The amplification of genetic determinants of plnA, plnJ and plnK has shown the presence of these genes in *L. plantarum* F12. The ability of the BLS from *L. plantarum* F12 to inhibit several pathogenic/spoilage bacteria and its characterization demonstrated its interest as a natural food preservative, in addition to its probiotic potential in prevention and treatment of infectious diseases.

Key words: *Lactobacillus plantarum*, plantaricin J/K, plantaricin A, bacteriocin-like substance, characterization.

INTRODUCTION

Lactic acid bacteria (LAB) have an important role in fermented food production because of their beneficial influence on nutritional, organic and shelf-life properties (Leroy and De Vuyst, 2004; Savadogo et al., 2006; Gillor et al., 2008). Many of LAB produce an array of antimicrobial substances such as organic acids, hydrogen peroxide, diacetyl, antifungal substances, reuterin and bacteriocins (De Vuyst and Leroy, 2007; Reis et al., 2012).

Lactobacilli belong to LAB group, are ubiquitous and found in gastrointestinal tract in healthy human beings (Holzapfel et al., 1998; Hsieh et al., 2008). Many species of *Lactobacillus* are known to produce bacteriocins which can inhibit the growth of other bacteria including spoilage and pathogen organisms (De Vuyst and Leroy, 2007; Todorov, 2008; Martinez et al., 2013). Lactobacilli have received a considerable attention for their great potential as natural preservatives in food materials (Savadogo et al., 2006; Diep et al., 2009; Lavilla et al., 2013) and they have also been investigated with regard to their probiotic traits (Kiliç et al., 2013; Bergillos-Meca et al., 2015). The

*Corresponding author. E-mail: fa.bahri@gmail.com. Tel: +213664712958.

Author(s) agree that this article remains permanently open access under the terms of the Creative Commons Attribution License 4.0 International License

probiotics are defined as "live microorganisms which when administered in adequate amounts confer health benefits for the host" (FAO/WHO, 2002). Bacteriocins produced by probiotic strains might play a role during *in vivo* interactions occurring in the human gastrointestinal tract, hence contributing to gut health (De Vuyst and Leroy, 2007; Corr et al., 2009). Actually, there is intriguing potential on the use of bacteriocigenic probiotics as pharmabiotics and/or novel alternatives to existing antibiotics (Gillor et al., 2008; Dobson et al., 2012; Arthur et al., 2014).

Bacteriocins are ribosomally-synthesized peptides or proteins with antimicrobial activity, produced by different groups of bacteria (Klaenhammer, 1988; Gàlvez et al., 2007). Bacteriocins from LAB are of low molecular weight antimicrobial peptides**.** They constitute a large and heterogeneous group of bacteriocins and varied in their peptide size, post-translation modifications, chemical stability and mechanism of action (Klaenhammer 1993; Drider et al., 2006). The LAB bacteriocins have been classified into three classes according to their biochemical and genetic properties: lanthionine (class 1), small, heat-stable, non lanthionine peptides (class II) and large heat-labile proteins (class III). The class II is divided into four subclasses. The subclass IIb called two peptide complexes represents the bacteriocins in which antimicrobial activity depends on the complementary action of the two different peptides (Heng et al., 2007)**.** The genes encoding the two different peptides are also genetically closely associated, being encoded in the same operon such as plantaricin J and K (Diep et al., 1996, Diep et al., 2009; Nissen-Meyer et al., 2010).

Several *Lactobacillus plantarum* strains have been isolated from fermented foods and numerous bacteriocins have been characterized for their use as natural food preservatives (Daeschel et al., 1990; Todorov, 2009; Gong et al., 2010; Lavilla et al., 2013). However, to the best of the authors' knowledge, there are few reports on bacteriocins produced by *L. plantarum* from human origin. Recently, the bacteriocins of human lactobacilli strains isolated as probiotics have gained a great momentum, due to their both potential use as biological preservatives and therapeutic antibiotics (Gillor and Ghazaryan 2007; Cotter et al., 2013; Das and Goyal, 2014).

The term "bacteriocin-like substance" is applied to antagonistic substances which are not completely defined or do not fit the typical criteria of bacteriocins. The BLS have been reported to inhibit a wide range of both Gram-positive and negative bacteria as well as fungi (McGroarty, 1993). In the present study, the characterization of physicochemical properties of BLS from *L. plantarum* F12 and the screening of the plantaricin A, J and K genes in this strain were reported. *L. plantarum* F12 was previously, isolated from faeces of Algerian healthy children and screened for its probiotic traits (Bahri et al., 2014).

MATERIALS AND METHODS

Bacterial strains and growth conditions

L. plantarum F12 strain was isolated from faeces of Algerian children for its probiotic traits and identified by phenotypic and genotypic methods (Bahri et al., 2014). The strain was cultured in Man Rogosa Sharp (MRS) medium at 37°C and stored at -80ºC and left in MRS broth, supplemented with 40% (v/v) glycerol.

The following bacteria were used to detect the BLS inhibitory activity: *Staphylococcus aureus* ATCC 29222, *Salmonella typhimurium*, *Salmonella infantis*, *Escherichia coli* ATCC 25122, *Shigella dysenteriae, Shigella sonnei, Escherichia coli*, *Enterococcus faecalis, Pseudomonas aeruginosa, Listeria monocytogenes, Listeria innocua, Bacillus cereus, Staphylococcus aureus, Lactobacillus rhamnosus, Lactobacillus plantarum, Lactobacillus curvatus, Lactobacillus acidophilus, Lactobacillus sake* and *Streptococcus thermophilus*. The strains *L. plantarum* LMG6804 and *Streptococcus thermophilus* were studied to detect the plantaricins genes. All strains were obtained from the culture collection CWBI of the Bio-Industries Unit (Gembloux AgroBiotech, University of Liege, Belgium). Bacterial strains were stored at -80°C in culture broth with glycerol (40%). Before experimental use, *L. plantarum* F12 strain and the indictor bacteria were subcultured twice and incubation was carried out at 37°C for 48 h.

Bacteriocin-like substance assay

Preparation of culture supernatant

To prepare *L. plantarum* F12 supernatant, the strain was grown overnight in the MRS broth at 37°C. After that, 10% of the subculture obtained was seeded in 1000 ml MRS broth and incubated for 72 h at 37°C. Then, cells were separated by centrifugation (10.000 rpm for 20 min, at 4°C). The cell free supernatant was adjusted to pH 6.5 with 5 N NaOH to exclude the acidity effect due to the formation of organic acids. The neutralized supernatant was treated with catalase from Sigma (5 mg/ml) to eliminate the effect of hydrogen peroxide (H_2O_2) and then sterilized by filtrating through cellulose acetate filter (0.2 µm pore size) (Daba et al., 1991).

Determination of the bacteriocin-like substance inhibitory spectrum

Bacteriocin-like substance activity of neutralized cell free supernatant, treated with catalase (NFSC) was tested by agar well diffusion assay (WDA) as described by Barefoot and Klaenhammer, (1983). Aliquots of 60 µl from NFSC were placed in wells (6 mm) cut in molten agar previously seeded with indicator strains. After 4 h at 4°C, the plates were incubated without aeration, at 30°C for LAB indicator and aerobically, at 37°C, for the other indictor strains for 18 to 24 h. Inhibition of growth was determined by the presence of an inhibition zone surrounding each agar well.

Determination of the bacteriocin-like substance titre

E. faecalis was used to determine the BLS titre. Two fold serial dilutions of NFSC were prepared. The titres of the BLS were quantified by the method described above. The antimicrobial activity of bacteriocin-like substance, expressed in activity units (AU) per milliliter, was defined as the reciprocal of the highest dilution showing a clear zone of growth inhibition of the indicator strain (Todorov and Dicks, 2005).

Table 1. PCR primers and conditions used for detection of bacteriocin gene fragments.

Targeted genes	Primers*	Sense	Expected size (bp)	Annealing temperature (°C)	Reference
Plantaricin (A)	5'GTACAGTACTAATGGGAG 3' 5'CTTACGCCAATCTATTATACG 3'	Forward Reverse	450	53	Remiger et al. (1996)
Plantaricin (J)	5'TAACGACGGATTGCTCTG 3' 5'AATCAAGGAATTATCACATTAGTC 3'	Forward Reverse	475	51	Rojo-Bezares et al. (2007)
Plantaricin (K)	5'AATCGCAGTGACTTCCAGAAC 3' 5'AGAGCAATCCGTCGTTAATAAATG 3'	Forward Reverse	469	53,7	Rojo-Bezares et al. (2007)

*Synthetic primers sequences were collected from Europeen Molecular Biology Laboratory (EMBL) and have been supplied by SIGMA-PROLIGO.

Characterization of the bacteriocin-like substance

Sensitivity to heat

To determine the effect of temperature on the BLS, the method of Barefoot and Klaenhammer, (1983) was performed with slight modifications. 1 ml of the NFSC was exposed to various heat treatments: 40, 60, 80, 100 and 121°C. Aliquot volumes of each fraction were then removed after 10, 30, 60 and 90 min. The residual activity of the BLS against *E. faecalis* was assayed by the WDA method.

pH sensitivity

The sensitivity of the BLS to pH variations was estimated by adjusting the pH of NFSC samples (5 ml) to pH 2, 4, 5, 6, 7, 8, 9, 10, 11 and 12 with 1 M HCl and 1 M NaOH. The samples were incubated for 4 h at room temperature. Then, they were neutralized to pH 6.5 to remove the effect of pH. The remaining bacteriocin-like substance activity was assayed against *E. faecalis* by the method described above.

Storage stability

The NFSC was stored at -80, -20, 4 and 37°C at different time intervals for four months. Samples were taken from the stored material to determine the BLS activity (Ten Brink et al., 1994) using *E. faecalis* by the WDA method.

Enzyme treatments

The sensitivity of the BLS to various enzymes was tested with the following enzymes: trypsin (Fluka), α-amylase (Fluka), lipase (Sigma), proteinase K (Sigma), pronase (Merck). The enzymes were added to the NFSC at a final concentration of 1.0 mg/ml. Samples were incubated for 1 h, 30 min at 37°C (Jack et al., 1996). The residual activity of BLS was performed as previously mentioned.

Detecting genes of plantaricin A and plantaricin J/K by polymerase chain reaction

The presence of plantaricin gene (A) and plantaricin cluster genes J/K were screened in the bacteriocin producer *L. plantarum* F12. The Wizard® genomic DNA purification kit (Promega, Madison, USA) was used to isolate the total DNA from the liquid culture of bacteria. To amplify and sequence plantaricin genes, the polymerase chain reaction (PCR) was carried out using the total bacterial DNA as a template. The PCR was prepared using specific primers and conditions (Table 1). After amplification, the PCR products were separated by electrophoresis in 1% (w/v) agarose gels, visualized by ethidium bromide staining then purified with the Microcon YM-100 kit (Bedford, MA, USA) according to the manufacturer's instructions. Universal primers and BigDye Terminator v3.0 kit were used for the sequencing of amplicons and the nucleotide sequences analysis was performed using the Vector NTI (Version 8) software package (BD Biosciences, San Jose, USA). *S. thermophilus* DNA and *L. plantarum* LMG6804 DNA were used as a negative and a positive control, respectively.

Statistical analysis

The data were calculated with mean values, and standard deviations (mean±SD) were determined from triplicate trials. Statistical significance of the results was evaluated by analysis of variance (ANOVA). Statistical significance was attributed to $P<0.05$.

RESULTS

Determination of bacteriocin-like substance inhibitory spectrum

The BLS of *L. plantarum* F12 displayed a large spectrum of antimicrobial activity. It inhibited 16 of the 19 indicator strains. All pathogenic bacteria tested were inhibited by the BLS except *P. aeruginosa.* The spoilage and pathogenic bacteria *L. monocytogenes*, *L. innocua* and *B. cereus* were strongly inhibited by the BLS. However, there was no bacteriocigenic activity against the LAB *L. rhamnosus* and *L. sake* (Table 2).

Determination of the bacteriocin-like substance titre

The BLS titre from *L. plantarum* F12 was performed by WDA method against *E. faecalis.* It showed an antimicrobial activity of 51000 AU/ml.

Table 2. Inhibitory spectrum of neutralized cell free supernatant, treated with catalase of *L. plantarum* F12.

Indicator organisms	Sensitivity
Escherichia coli ATCC 25122	+++
Escherichia coli	++
Salmonella infantis	+++
Salmonella typhimurium	+++
Shigella dysenteriae	+
Shigella sonnei	++
Enterococcus faecalis	+++
Pseudomonas aeruginosa	-
Staphylococcus aureus ATCC 29222	+++
Staphylococcus aureus	++
Listeria monocytogenes	+++
Listeria innocua	+++
Bacillus cereus	+++
Lactobacillus rhamnosus	-
lactobacillus plantarum	+
Lactobacillus curvatus	++
Lactobacillus acidophilus	++
Lactobacillus sake	-
Streptococcus thermophilus	++

- No inhibition zone; +, 5 mm < zone < 10 mm; ++, 10 mm < zone < 15 mm; +++, zone >15 mm. Each data point is the average of repeated measurements from 03 independently replicated experiments, n = 3. P<0.05.

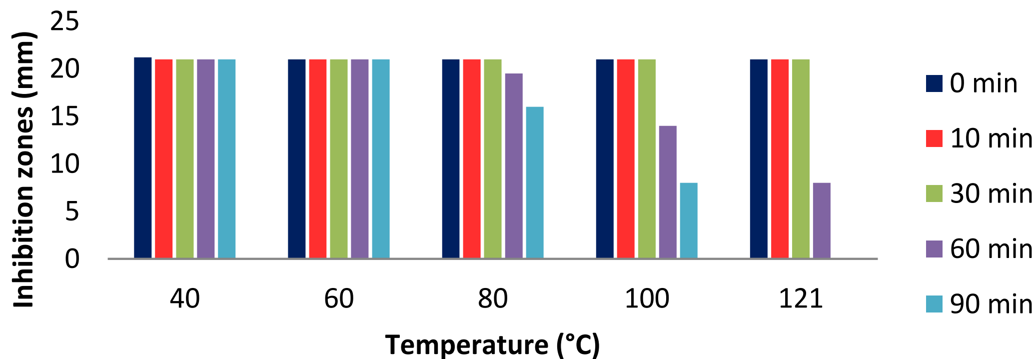


Figure 1. Effect of temperature variation on bacteriocin-like substance from L. plantarum F12. Each data point is the average of repeated measurements from 03 independently replicated experiments, n = 3. P<0.05.

Characterization of the bacteriocin-like substance

The BLS was heat stable at different temperatures (40 - 121°C) for 30 min. After this time, there was a decline in its activity as observed with prolonged treatment. However, there was no activity after 90 min at 121°C as shown in Figure 1.

The BLS activity was stable at pH values ranging from 2 to 8. Its activity started to decrease at pH 9 and it became completely inactive at Ph 12 (Figure 2). Regarding the effect of storage (Figure 3), the BLS maintained its total activity at -80°C for 120 days and at -20°C for 60 days. However, a decrease on its stability was observed at 4°C for 30 to 120 days and at 37°C for 30 days. After 60 days of storage, the BLS was completely inactivated at 37 °C.

The treatment with trypsin, proteinase k and pronase led to a total loss of activity of the BLS. However, α-amylase and lipase do not affect the BLS activity (Figure 4).

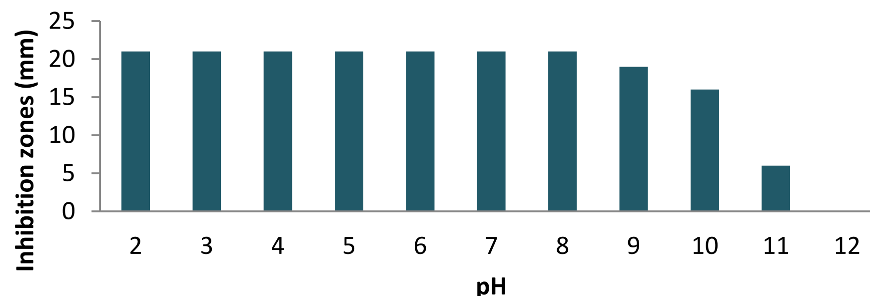


Figure 2. Effect of pH variation on the activity of bacteriocin-like substance from *L. plantarum* F12. Each data point is the average of repeated measurements from 3 independently replicated experiments, n = 3. P<0.05.

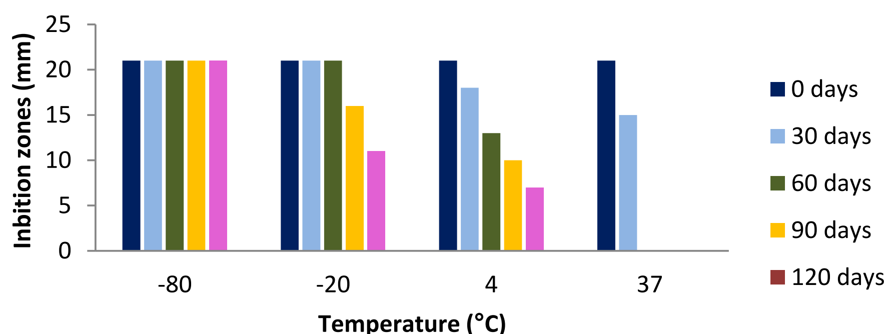


Figure 3. Effect of storage on the activity of bacteriocin-like substance of *L. plantarum* F12. Each data point is the average of repeated measurements from 3 independently replicated experiments, n = 3. P<0.05.

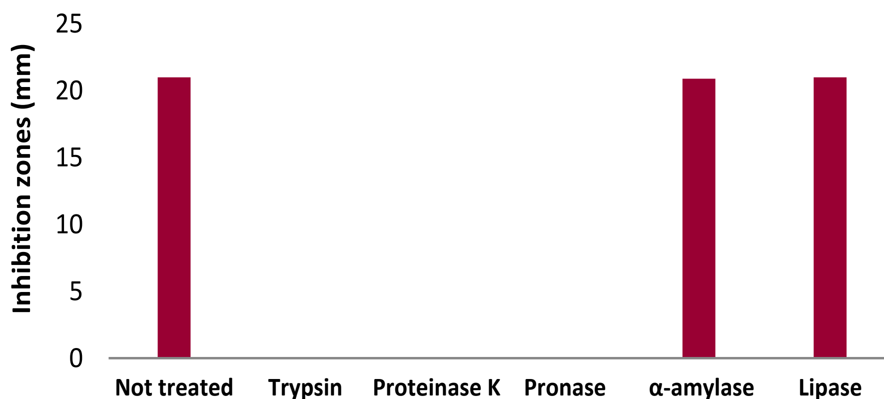


Figure 4. Effect of enzyme treatments on the activity of bacteriocin-like substance of *L. plantarum* F12. Each data point is the average of repeated measurements from 3 independently replicated experiments, n = 3. P<0.05.

Detecting genes of plantaricin A and plantaricin J/K by PCR

Results of PCR amplification of genomic DNA, performed in order to detect the genes of bacteriocins from *L. plantarum* F12, are shown in Figure 5. It was found that the genes plnA, plnJ and plnK, observed in the wells 1, 2 and 3 respectively, were presented in this strain.

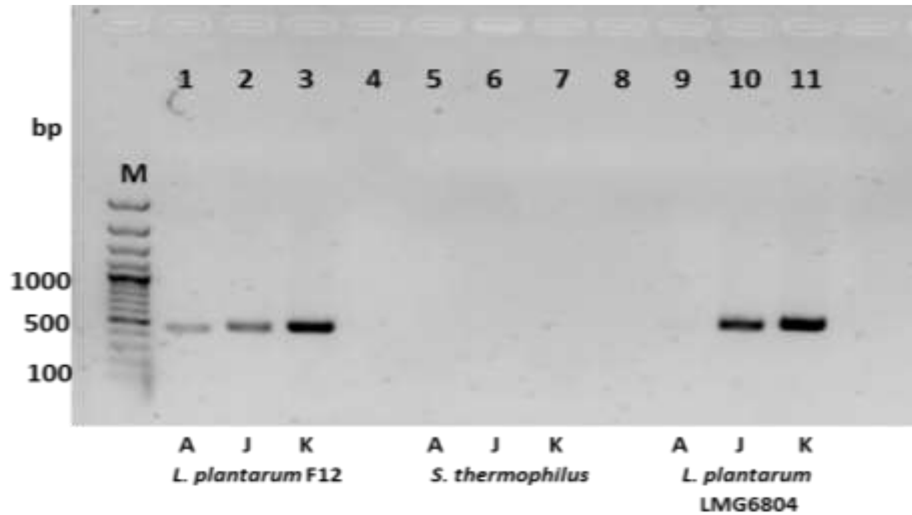


Figure 5. Agarose gel showing results from the genomic DNA PCR amplification from *L. plantarum* F12 using specific primer pairs for plnA; plnJ and plnK observed in the wells 1, 2 and 3 respectively. *S. thermophilus* DNA and *L. plantarum* LMG6804 DNA were used as a negative and a positive control, respectively.

DISCUSSION

In this study, preliminary characterizations were carried out by using NFSC prepared from probiotic *L. plantarum* F12. The NFSC was active against several indictor bacteria that will be discussed sooner. The antimicrobial activity of NFSC was not due to acidity or hydrogen peroxide. So, the activity was neither lost after neutralizing of pH value nor after treatment with catalase. In otherwise, the total loss of the antagonistic effect of NFSC with the all proteolytic enzymes (trypsin, proteinase K and pronase) indicates its proteinaceous nature. The other enzymes tested (α-amylase and lipase) did not cause any inactivation suggesting the lack of lipid or carbohydrate moiety in the NFSC. According to those results, the antimicrobial compound(s) contained in NFSC of *L. plantarum* F12 could be considered as bacteriocins or BLS. Since this (these) compound(s) have not yet been characterized on amino acids and nucleotides sequences, they will be referred to as BLS (McGroarty, 1993; Corsseti et al., 2004).

The BLS from *L. plantarum* F12 displayed a large spectrum of antimicrobial activity. It inhibited numerous indicator strains including pathogenic and spoilage bacteria such as *Escherichia coli, Salmonella infantis, Salmonella typhimurium*, *Shigella sonnei, Staphylococcus aureus, Listeria monocytogenes, Listeria innocua, Bacillus subtilis* and some lactobacilli sp. Depending on the strains, *Lactobacillus* is known to produce BLS which are active against different bacterial species (Abo-Omer, 2007; Ghanbari et al., 2013, Anacarso et al., 2014; Sabia et al., 2014). Recently, there was a greatest need of more natural and safe food products. For this purpose, the bacteriocinogenic compounds provide an alternative to reduce chemical preservatives and intensity of heat treatment in food preservation (Gàlvez et al., 2007). So, BLS of LAB may offer a wide applicability in food biocontrol because of their antimicrobial broad spectrum against both pathogens and spoilage bacteria.

The titration of the BLS of *L. plantarum* F12 showed a high antimicrobial activity (51000 AU/ml). Our result fall in the range of Diop et al. (2007), where titration value is between 10^4 and 10^5. However, this is higher than that of the study carried by Todorov in 2008 (30000 AU/ml) and Ogunbanwo et al. in 2003 (6400 AU/ml).

For its application as biopreservative, the bacteriocin must maintain its activity in thermally processed food. In general, plantaricins are high thermoresitant (Todorov and Dicks, 2005; Rojo-Bezares et al., 2007; Martinez et al., 2013). But, some of them were reported to be less heat stable such as plantaricin TF711 that retained only 70% of its activity after boiling and non activity upon autotclaving (Hernandez et al., 2005). The BLS of *L. plantarum* F12 was stable at high temperatures and autoclaving. According to Todorov (2009), bactriocins of class II are heat resistant up to temperatures 100°C,or autoclavable, through this, we can supposed our BLS belongs to class II.

About pH variations, several bacteriocins were found to be highly stable at acidic conditions and many of them are inactivated under alkaline pH values (Ten Brink et al., 1994; Hernandez et al., 2005; Todorov and Franco 2010). This is in line with our study; the BLS of *L. plantarum* F12 remained active in acidic pH range from 2 to 6, but its activity decreased at pH 9 until its full inactivation at pH 12. While, some bacteriocins remained

active in acidic, neutral and alkaline conditions such as bacteriocin ST71KS (Martinez et al., 2013).

Another criterion required for the use of bacteriocins in food industry is their stability during storage. The antimicrobial activity of the BLS from *L. plantarum* F12 was maintained in frozen state at -80°C for four months and at -20°C for two months. The bacteriocins stability at low temperature for a long duration allows their use in refrigerated and frozen foods in order to prevent the pathogenic and spoilage psycrotrophic micro-organisms growth (Hernandez et al., 2005).

According to Diep et al. (1996), plnJ and plnK genes coded respectively for plantaricine J and plantaricine K; two small cationic bacteriocin-like peptides belonging to the subclass IIb. The plnA induces transcription of genes organized in the operon of plnJKLR which encompass the gene pair of pln JK (Diep et al., 1996, 2009). *L. plantarum* F12 harbors the bacteriocin genes plnJ, plnK and plnA. The presence of these genes in *L. plantarum* F12 assumes that the BLS produced by this strain can be encoded by these genes and belongs to the subclass IIb.

Conclusion

L. plantarum F12, a probiotic strain isolated from human samples, produced a BLS which could interest the food industry as a biopreservative and medical sector as a pharmabiotic. It displays a strong antimicrobial activity against pathogens and spoilage bacteria, is stable to heat treatment and storage and active on a wide pH range. However, further studies were needed to get more characterizations of this BLS.

Conflict of interest

The authors have not declared any conflict of interest

ACKNOWLEDGEMENTS

A part of this research was conducted at the University of Liege/Gembloux Agro Bio-tech/ Centre Wallon de Biologie Industrielle (CWBI), Belgium. The authors deeply acknowledge Professor Philippe Thonart and the team of CWBI, for their valuable technical assistance.

REFERENCES

Abo-Omer AE (2007). Characterization of a Bacteriocin-Like Inhibitory Substance Produced by Lactobacillus plantarum Isolated from Egyptian Home-Made Yogurt. Science Asia 33:313-319.

Anacarso I, Missi P, Condo C, Issepi R, Bondi M, Sabia C, De Niederhausern S (2014). A bacteriocin-like substance produced from *Lactobacillus pentosus* 39 is a natural antagonist for the control of *Aeromonas hydrophila* and *Listeria monocytogenes* in fresh salmon fillets. Food. Sci. Technol. 55(2):604-611.

Arthur TD, Cavera VL, Chikindas ML (2014). On bacteriocin delivery systems and potential applications. *Future Microbiol.* 9(2): 235-248.

Bahri F, Lejeune A, Dubois-Dauphin R, Elmejdoub T, Boulahrouf A, Thonart P (2014). Characterization of *Lactobacillus* strains isolated from Algerian children faeces for their probiotic properties. Afr. J. Mirobiol. Res. 8(3):297-303.

Barefoot SF, Klaenhammer TR (1983). Detection activity of lacticin B, a bacteriocin produced by *Lactobacillus acidophilus*. Appl. Environ. Microbiol. 45(6):1808-1815.

Bergillos-Meca T, Costabile A, Walton G, Moreno-Montoro M, Ruiz-Bravo A, Ruiz-Lopez MR (2015). In vitro evaluation of the fermentation properties and potential probiotic activity of *Lactobacillus plantarum* C4 in batch culture systems. Food. sci. technol. 60:420-426.

Corr SC, Hill C, Gahan CGM (2009). Understanding the mechanisms by which probiotics inhibit gastrointestinal pathogens. *Adv. Food. Nutr. Res.* 56:1-15.

Corsseti A, Settanni L, Van Sinderen D (2004). Characterization of bacteriocin-like inhibitory substances (BLIS) from sourdough lactic acid bacteria and evaluation of their in vitro and in situ activity. *J. Appl. Microbiol.* 96(3):521-34

Cotter PD, Ross RP, Hill C (2013). Bacteriocins - a viable alternative to antibiotics? Nat. Rev. Microbiol. 11: 95-105.

Daba H, Pandian S, Gosselin JF, Simard RE, Huang J, Lacroix C (1991). Detection and activity of bacteriocin produced by *Leuconostoc mesenteroides*. Appl. Environ. Microbiol. 57:3450-3455.

Daeschel MA, McKinney MC, McDonald LC (1990). Bactericidal activity of *Lactobacillus plantarum* C11. Food Microbiol. 7:91-99.

Das A, Goyal A (2014). Characterization of a noncytotoxic bacteriocin from probiotic *Lactobacillus plantarum* DM5 with potential as a food preservative. Food Funct. 5:2453-2462.

De Vuyst L, Leroy F (2007). Bacteriocins from Lactic Acid Bacteria: Production, Purification, and Food Applications. *J. Mol. Microbiol. Biotechnol.* 13:194-199.

Diep DB, Havarstein LS, Nes IF (1996). Characterization of the Locus Responsible for the Bacteriocin production in *Lactobacillus plantarum* C11. J. Bacteriol. 178:4472-4483.

Diep DB, Straume D, Kjos M, Torres C, Nes IF (2009). An overview of the mosaic bacteriocin pln loci from *Lactobacillus plantarum*. *Peptides.* 30: 1562-1574.

Diop MB, Dubois-Dauphin R, Tine E, Ngom A, Destain J, Thonart P (2007). Bacteriocin producers from traditional food products. *B.A.S.E.* 11: 275-328.

Dobson A, Cotter PD, Ross RP, Hill C (2012). Bacteriocin Production: a Probiotic Trait? Appl. Environ. Microbiol. 78:1-6.

Drider D, Fimland G, Héchard Y, McMullen LM, Prévost H (2006). The continuing story of class IIa bacteriocins. Microbiol. Mol. Biol. Rev. 2: 564-582.

FAO/WHO (2002). Guidelines for the evaluation of probiotics in food. Food and Agriculture Organization of the United Nations and World Health Organization (Organisation Mondiale pour la Santé OMS).Working Group Report. London, Ontario, Canada.

Gálvez A, Abriouel H, López RL, Ben Omar N (2007). Bacteriocin-based strategies for food biopreservation. Int. J. Food. Microbiol. 120: 51-70.

Ghanbari M, Jami M, Kneifel W, Domig KJ (2013) Antimicrobial activity and partial characterization of bacteriocins produced by lactobacilli isolated from sturgeon fish. Food. control. 32: 379-385.

Gillor O, Ghazaryan L (2007). Recent advances in bacteriocin application as antimicrobials. Recent Patent on Anti-infective Drug Discov. 2: 115-122.

Gillor O, Etzion A, Riley MA (2008). The dual role of bacteriocins as anti- and probiotics. Appl. Microbiol. Biotechnol. 81(4): 591-606.

Gong HS, Meng XC, Wang H (2010). Plantaricin MG active against Gram negative bacteria produced by *Lactobacillus plantarum* KLDS1.0391 isolated from "Jiaoke", a traditional fermented cream from China. Food Control. 21:89-96.

Heng NCK, Wescombe PA, Burton JP, Jack RW, Tagg JR (2007). The diversity of bacteriocins in Gram positive bacteria. In: Riley, MA.; Chavan, M., editors. Bacteriocins: ecology and evolution. Springer; Berlin: pp. 45-92.

Hernandez D, Cardell E, Zarate V (2005). Antimicrobial activity of lactic acid bacteria isolated from Tenerife cheese: initial characterization of

plantaricin TF711, a bacteriocin-like substance produced. J. App. Microbiol. 99:77-84.

Holzapfel WH, Haberer P, Snel J, Schillinger U, Huisin't Veld JHJ (1998). Overview of gut flora and probiotics. Int. J. Food. Microbiol. 41:85-101

Hsieh MH, Versalovic J (2008). The Human Microbiome and Probiotics: Implications for Pediatrics. Curr. Probl. Pediatr. Adolesc. Health. Care. 38:309-327.

Jack RW, Wan J, Gordon , Harmark K, Davidson BE, Hillier AJ, Wettenhall REH, Hickey MW, Coventry MJ (1996). Characterization of the chemical and antimicrobial properties of piscicolin 126, a bacteriocin produced by *Carnobacterium piscicola* JG 126. Appl. Environ. Microbiol. 62:2897-2903.

Kılıç GB, Kuleaşan H, Sömer VF, Akpınar D (2013). Determining potential probiotic properties of human originated *Lactobacillus plantarum* strains. Biotechnol. Bioprocess. Eng. 18:479-485.

Klaenhammer TR (1988). Bacteriocins of lactic acid bacteria. Biochimie. 70:337-349.

Klaenhammer TR (1993). Genetics of bacteriocins produced by lactic acid bacteria. FEMS. Microbiol. Rev. 12:39-86.

Lavilla-Lerma L, Pérez-Pulido R, Martínez-Bueno M, Maqueda M, Valdivia E (2013). Characterization of functional, safety, and gut survival related characteristics of *Lactobacillus* strains isolated from farmhouse goat's milk cheeses. Int. J. Food. Microbiol. 163:136-145.

Leroy F, De Vuyst L (2004). Lactic acid bacteria as functional starter cultures for the food fermentation industry. Trends. Food. Sci. Technol. 15(2):67-78.

Martinez RCR, Wachsman M, Torres NI, LeBlanc JG, Todorov SD, Franco BDGM (2013). Biochemical, antimicrobial and molecular characterization of a noncytotoxic bacteriocin produced by *Lactobacillus plantarum* ST71KS. Food. Microbiol. 34:376-381.

McGroarty J (1993). Probiotic use of lactobacilli in the human female urogenital tract. FEMS Immunol. Med. Microbiol. 6:251-264.

Nissen-Meyer J, Oppegard C, Rogne P, Haugen HS, Kristiansen PE (2010). Structure and mode of action of the two-peptide (class-IIb) bacteriocins. Probiotics. Antimicrob. Proteins. 2:52-60.

Ogunbanwo ST, Sanni AI, Onilude AA (2003). Characterization of bacteriocin produced by *Lactobacillus plantarum* F1 and *Lactobacillus brevis* OGI. Afr. J. Biotechnol. 2:219-227.

Reis JA, Paula AT, Casarotti SN, Penna ALB (2012). Lactic Acid Bacteria Antimicrobial Compounds : Characteristics and Applications. Food. Eng. Rev. 4:124-126.

Remiger A, Ehrmann MA, Vogel RF (1996). Identification of bacteriocin genes in Lactobacilli by polymerase chain reaction (PCR). Syst. Appl. Microbiol. 19:28-34.

Rojo-Bezares B, Saenz Y, Navarro L, Zarazaga M, Ruiz-Larrea F, Torres C (2007). Coculture-inducible bacteriocin activity of *Lactobacillus plantarum* strain J23 isolated from grape must. Food. Microbiol. 24:482-491

Sabia C, Anacarso I, Bergonzini A, Gargiulo R, Sarti M, Condo C, Missi P, De Niederhausern S, Issepi R, Bondi M (2014). Detection and partial characterization of a bacteriocin-like substance produced by *Lactobacillus fermentum* CS57 isolated from human vaginal secretions. Anaerobe 26:41-45.

Savadogo A, Ouattara Cheik AT, Bassole Imael HN, Traore SA (2006). Bacteriocins and lactic acid bacteria - a minireview. Afr. J. Biotechnol. 5(9):678-683

Ten Brink B, Minekus M, Vander Vossen JMBM, Leer RJ, Huis JHJ (1994). Antimicrobial activity of lactobacilli: preliminary characterization and optimisation of production of acidocin B., a novel bacteriocin produced by *L. acidophilus* M46. J. Appl. Bacteriol. 77:140-148.

Todorov SD, Dicks LMT (2005). *Lactobacillus plantarum* isolated from molasses produces bacteriocins active against Gram negative bacteria. Enzyme Microb. Technol. 36:318-326.

Todorov SD (2008). Bacteriocin production by *Lactobacillus plantarum* AMA-K isolated from amasi, a zimbabwean fermented milk product and study of the adsorption of bacteriocin ama-k to *Listeria* sp. Braz. J. Microbiol. 39:178-187.

Todorov SD (2009). Bacteriocin from *Lactobacillus plantarum* – Production, Genetic organization and mode of action. Braz. J. Microbiol. 40:209-221.

Todorov SD, Franco BDGM (2010). *Lactobacillus plantarum*: characterization of the species and application in food protection. Food Res. Int. 26:205-229.

academicJournals

Vol. 10(43), pp. 1806-1812, 21 November, 2016
DOI: 10.5897/AJMR2016.8333
Article Number: 131AE5661659
ISSN 1996-0808
Copyright © 2016
Author(s) retain the copyright of this article
http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Full Length Research Paper

Effectiveness assessment of mouthwashes formulated from the essential oils of some beninese medicinal plants against oral germs

Jacob Bonou[1,2], Farid Baba-Moussa[1,2], Pacôme Agossou Noumavo[1,3], Adonice Zannou[2], Diane Aloukoutou[2], Lisette Kpavode[2], Hélène Ahouandjinou[1,2], Joachim Djimon Gbénou[4], Fatiou Toukourou[1] and Lamine Baba-Moussa[3*]

[1]Laboratoire de Microbiologie et des Technologies Alimentaires, Département de Biologie Végétale, Faculté des Sciences et Techniques, Université d'Abomey-Calavi, 01 BP 526 Cotonou, Bénin.
[2]Laboratoire National de Contrôle de Qualité des Médicaments et Consommables Médicaux, Ministère de la Santé, 06 BP139 Cotonou Bénin.
[3]Laboratoire de Biologie et de Typage Moléculaire en Microbiologie, Département de Biochimie et de Biologie Cellulaire, Faculté des Sciences et Techniques, Université d'Abomey-Calavi, 05 BP 1604 Cotonou, Bénin.
[4]Laboratoire de Pharmacognosie et des Huiles Essentielles, Département de Chimie, Faculté des Sciences et Techniques, Université d'Abomey-Calavi, ISBA-Champ de foire Cotonou, Bénin.

Received 9 October, 2016 : Accepted 4 November, 2016

Faced with the increasing growth of microorganism's resistance to conventional antibiotics, the search of new bioactive molecules having pharmaceutical interest is more than ever committed. This study aimed to evaluate *in vitro* the effectiveness of seven mouthwashes formulated from essential oils of *Ocimum gratissimum*, *Ocimum basilicum*, *Cymbopogon citratus*, *Clausena anisata*, *Lippia multifora*, *Eugenia caryophyllata* or *Mentha piperita* against five oral germs (*Micrococcus luteus* ATCC 10240, *Staphylococcus aureus* ATCC 29213, *Proteus mirabilis* ATCC 24974, *Pseudomonas aeruginosa* ATCC 27853 and *Candida albicans* IP 4872). The antimicrobial power of different mouthwashes was evaluated through the determination of their minimum inhibitory and bactericidal concentrations by microdilution method. All mouthwashes had an inhibitory and bactericidal effect against the studied germs. Their minimum inhibitory and bactericidal concentration varied according to the type of germ (from 0.125 to 1 µg/ml). The mouthwashes formulated from essential oils of *O. basilicum* and *C. citratus* were the most effective against all germs. They were followed by mouthwashes formulated from essential oils of *O. gratissimum* and *C. anisata.*

Key words: Medicinal plants, essential oils, antimicrobial activity, minimum inhibitory concentration, minimum bactericidal concentration, Mouthwash.

INTRODUCTION

The oral diseases appear among the most common diseases in the world because of their prevalence and their high impact. These diseases represent the third worldwide scourge after cardiovascular and cancer disease (Ameziane, 2014). Indeed, the dental caries and periodontal diseases induce the progressive destruction

of oral tissues, which may have the repercussions on their functions (phonation, mastication and swallowing) (Wheater et al., 2004) and on aesthetics and relationship skills of patients. These oral diseases are caused by some microorganisms (fungi and bacteria) of oral cavity and dental plaque. Indeed, a large number of micro-organisms coexist in the very complex oral environment (Peluchonneau, 2011). These microorganisms found in this medium, require nutrients for metabolism from food debris, desquamated cells and some saliva constituents.

There are a large variety of antibiotics and antiseptics against these dental germs. However, the conventional treatment of these diseases is extremely costly. In many low income countries, if the treatment of dental caries was available, the cost of treatment of a child exceeds the total health care budget spent on them (Yee et al., 2002). This situation forces the people to resort to traditional medicine. According to the World Health Organization, approximately 65 to 80% of developing countries population uses the medicinal plants for their health needs because of difficulty to access to modern medicine (Sekoussounon, 2012). These plants are used in decoction, maceration, infused or essential oils forms.

Thus, thanks to new extraction techniques, identification and characterization of organic molecules, over 25 to 50% of prescribed drugs today have for active constituents, the bioactive molecules of medicinal plants (Akibou, 2014). In Africa and Benin in particular, the exploration of active constituents from plants for therapeutic use focuses more and more the researcher's attention in recent years. In addition, the essential oils extracted from medicinal plants are easy to use and often effective (Quenum et al., 2003). Recent studies have shown that *Cymbopogon citratus*, *Clausena anisata*, *Ocimum gratissimum*, *Ocimum basilicum*, *Lippia multifora*, *Eugenia caryophyllata* and *Mentha piperita* have developed a very strong antimicrobial activity against many oral bacteria (Bonou et al., 2016a, b, c). The chemical compositions of essential oils extracted fron the precedent medicinal plants are shown in Table 1. In this work, seven mouthwashes based on previous essential oils were formulated and evaluated for effectiveness against five oral germs.

MATERIALS AND METHODS

Plant

The plant material consisted of the following medicinal plants: *O. gratissimum* (Lamiaceae), *O. basilicum* L. (Lamiaceae), *C. citratus* DC. Stapf (Poaceae), *C. anisata* W. (Rutaceae), *L. multifora* M. (Verbenaceae), *E. caryophyllata* (Myrtaceae) and *M. piperita* L (Lamiaceae). The plants were collected from southern Benin (West Africa) by Bonou et al. (2016). These plants are commonly used to treat the oral diseases by the population of studied area (Benin, West Africa). The essential oils used in this study were extracted from these plants using hydrodistillation method (Bonou et al., 2016a).

Microorganisms

Four bacterial strains including two Gram positive bacteria (*Micrococcus luteus* ATCC 10240 and *Staphylococcus aureus* ATCC 29213) and two Gram negative bacteria (*Proteus mirabilis* ATCC 24974 and *Pseudomonas aeruginosa* ATCC 27853) and one yeast (*Candida albicans* IP 4872) were used in this study. They were obtained from the Laboratory of Biology and Molecular Typing in Microbiology (University of Abomey, Benin) and National Quality Control Laboratory (Ministry of Health, Benin). They are kept in Muller Hinton broth supplemented with glycerol (10%) at -20°C.

Formulation of mouthwashes

The mouthwashes were formulated using conventional components. Hundred millimeters (100 mm) of mouthwash solution contained the essential oil (0.2 ml), saccharin (0.5 g), glycerol (0.4 g), sodium hydroxide (0.4 g), methyl salicylate (0.12 g), tween 60 (4 g), alcohol 96°C (4 ml) and sterile distilled water (100 ml). Each essential oil was used to formulate one mouthwash. The different mouthwashes are presented in Table 2.

Assessment of effectiveness of mouthwashes

The effectiveness of mouthwashes was evaluated by the determination of their minimum inhibitory and bactericidal concentrations against the microorganisms (*M. luteus* ATCC 10240, *S. aureus* ATCC 29213, *P. mirabilis* ATCC 24974, *P. aeruginosa* ATCC 27853 and *C. albicans* IP 4872) through the microdilution method using microplate of 12 wells (Yayi-Ladekan et al., 2011).

Determination of minimum inhibitory concentration of mouthwashes

The minimum inhibitory concentration is the lowest concentration for which there is no visible growth of studied microorganism. The determination of minimum inhibitory concentration of different mouthwashes was performed by microdilution method.

Preparation of microbial inoculum

The previous cultures of strains were centrifuged at 10,000 rpm for 10 min. The microbial pellets obtained were washed and suspended in Mueller Hinton broth (for bacteria) or Sabouraud (for yeast). The concentrations of these suspensions were adjusted to 10^8 CFU/ml (OD 1 at 600 nm) using a spectrophotometer (BIOMATE 3S, Thermo Scientific). The different suspensions were diluted to 10^6 CFU/ml (bacteria) or 10^7 CFU/ml (*C. albicans*).

*Corresponding author. E-mail: fbmouss@yahoo.fr. Tel: +229 96926828

Author(s) agree that this article remains permanently open access under the terms of the Creative Commons Attribution License 4.0 International License

Table 1. Chemical composition of essential oils extracted from some medicinal plants.

Plant species	Family	Compounds	Total (%)
Ocimum gratissimum	Lamiaceae	Thymol (30.62%), para-cymene (25.25%), gamma-terpinolene (24.24%), alpha-thujene (7.60%) and myrcene (6.56%).	94.27
Ocimum basilicum	Lamiaceae	Estragole (85.92%), trans-alpha-bergamotene (3.71%), para-méthoxy-cinnamaldehyde (2.03%) and 1,8-cineole (2.01%).	93.67
Cymbopogon citratus	Poaceae	Myrcene (11.48%), neral (33.53%), geranial (43.10%), geraniol (5.58%) and geranyl acetate (4.47%).	98,16
Clausena anisata	Rutaceae	Estragole (97.10%).	97.10
Lippia multiflora	Verbenaceae	Linalol (45,10%), 1,8-cineole (25,40%), Myrtenol (10,40%), α-terpineol (8,20%) and α-pinene (5,90%).	95,00
Eugenia caryophyllata	Myrtaceae	Eugenol (85.90%), eugenyl acetate (10.20%) and beta-caryophyllene (1.98%).	98.08
Mentha piperita	Lamiaceae	Menthone (38.50%), menthol (52.09 %), menthyl acetate (3.21%) and 1,8-cinéole (2.87%).	96.67

Bonou et al. (2016a, b, c)

Table 2. The different mouthwashes.

No	Code	Correspondence
1	Og	Mouthwash formulated from *Ocimum gratissimum*
2	Ob	Mouthwash formulated from *Ocimum basilicum*
3	Cc	Mouthwash formulated from *Cymbopogon citratus*
4	Ca	Mouthwash formulated from *Clausena anisata*
5	Lm	Mouthwash formulated from *Lippia multiflora*
6	Ec	Mouthwash formulated from *Eugenia caryophyllata*
7	Mp	Mouthwash formulated from *Mentha piperita*

Preparation of microplate

Nine hundred and fifty (950) microliters of Mueller Hinton broth or Sabouraud broth were distributed into each plate well. Fifty (50) microliters of the mouthwash were added to each well of first column and 950 µl to the wells of third column of microplate. Serial dilutions from the third column were perfomed well by well and line by line (series of half dilutions). All wells except those in first column were inoculated with 50 µl of a bacterial inoculum. Two control wells were performed.

The first control well contained the bath medium (950 µl) and mouthwash (50 µl) while the second control well contained the bath medium (950 µl) and microbial suspension (50 µl). Each line of microplate was used for one mouthwash. The microplate was covered and incubated at 37°C during 24 h (bacteria) or at 27°C during 48 h (*C. albicans*). The reading of result was made by comparing control wells and test wells.

Determination of minimum bactericidal concentration of mouthwashes

The minimum bactericidal concentration of tested mouthwashes was determined by sub culturing the content of all wells onto a Mueller Hinton (bacteria) or Sabouraud (yeast) agar and incubated at 37°C during 24 h for bacteria and 48 h for yeast. The lowest dilution of mouthwash that no visible microorganism growth on solid medium was taken as minimum bactericidal concentration (Farshori et al., 2013).

Data processing

The software Microsoft Office Excel 2010 was used to process the data specially to calculate the average values of minimum inhibitory and bactericidal concentration of each mouthwash according the different oral germs and to generated the figures.

Preparation of microplate

Nine hundred and fifty (950) microliters of Mueller Hinton broth or Sabouraud broth were distributed into each plate well. Fifty (50) microliters of the mouthwash were added to each well of first column and 950 µl to the wells of third column of microplate. Serial dilutions from the third column

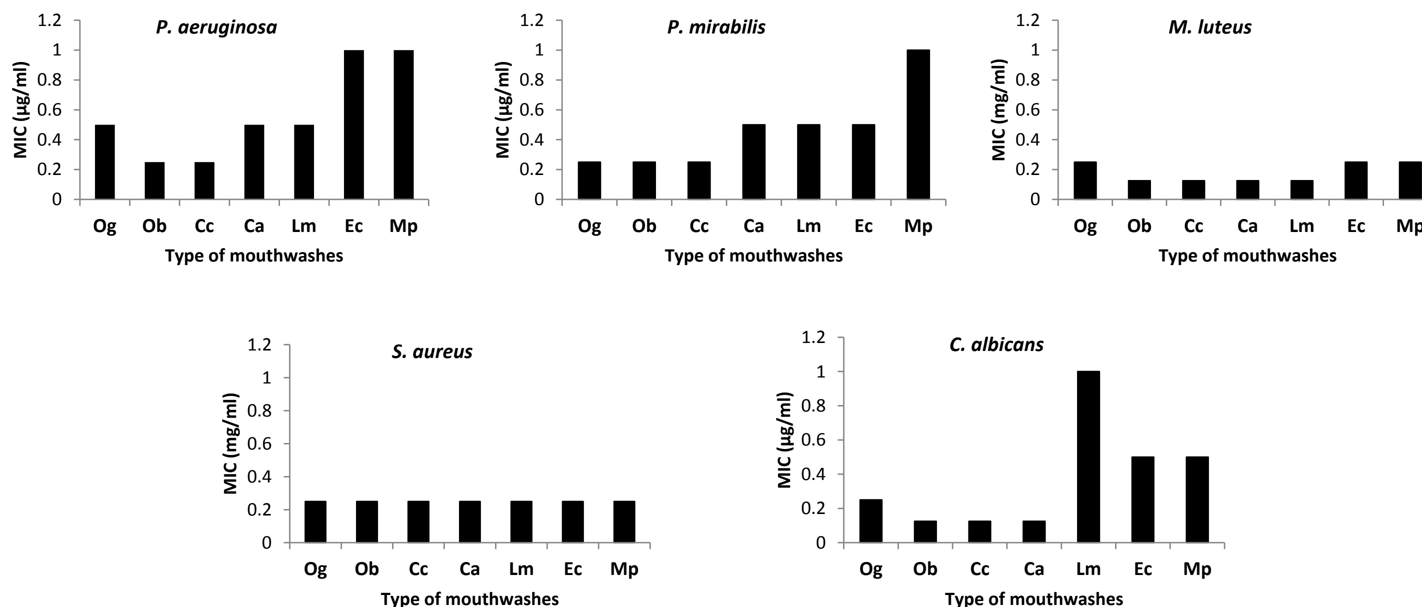


Figure 1. Minimum Inhibitory Concentration (MIC) of different mouthwashes against some reference strains. *P. aeruginosa = Pseudomonas aeruginosa; P. Mirabilis = Proteus mirabilis ; M. Luteus = Micrococcus luteus; S. Aureus = Staphylococcus aureus; C. Albicans = Candida albicans.* Og = Mouthwash formulated from *Ocimum gratissimum*; Ob = Mouthwash formulated from *Ocimum basilicum*; Cc = Mouthwash formulated from *Cymbopogon citratus*; Ca = Mouthwash formulated from *Clausena anisata*; Lm = Mouthwash formulated from *Lippia multiflora*; Ec = Mouthwash formulated from *Eugenia caryophyllata* and Mp = Mouthwash formulated from *Mentha piperita*.

microplate was covered and were perfomed well by well and line by line (series of half dilutions). All wells except those in first column were inoculated with 50 µl of a bacterial inoculum. Two control wells were performed. The first control well contained the bath medium (950 µl) and mouthwash (50 µl) while the second control well contained the bath medium (950 µl) and microbial suspension (50 µl). Each line of microplate was used for one mouthwash. The incubated at 37°C during 24 h (bacteria) or at 27°C during 48 h (*C. albicans*). The reading of result was made by comparing control wells and test wells.

Determination of minimum bactericidal concentration of mouthwashes

The minimum bactericidal concentration of tested mouthwashes was determined by sub culturing the content of all wells onto a Mueller Hinton (bacteria) or Sabouraud (yeast) agar and incubated at 37°C during 24 h for bacteria and 48 h for yeast. The lowest dilution of mouthwash that no visible microorganism growth on solid medium was taken as minimum bactericidal concentration (Farshori et al., 2013).

Data processing

The software Microsoft Office Excel 2010 was used to process the data specially to calculate the average values of minimum inhibitory and bactericidal concentration of each mouthwash according the different oral germs and to generated the figures.

RESULTS AND DISCUSSION

Inhibitory effect of different mouthwashes against germs growth

The antimicrobial activity of mouthwashes formulated from essential oils of *O. gratissimum*, *O. basilicum*, *C. citratus*, *C. anisata*, *L. multiflora*, *E. caryophyllata* and *M. piperita* tested *in vitro* against two Gram positive bacteria (*M. luteus*, *S. aureus*), two Gram negative bacteria (*P. aeruginosa*, *Proteus mirabilis*) and one yeast (*C. albicans*) revealed interesting results. Indeed, all mouthwashes have developed antimicrobial

activity against all germs (Figure 1).

The minimum inhibitory concentrations of the mouthwashes are varied according the type of germ and ranged from 0.125 µg/ml to 1 µg/ml. The mouthwashes formulated from essential oils of *O. basilicum* and *C. citratus* were most effective against *P. aeruginosa* with a minimum inhibitory concentration of 0.25 µg/ml. In contrary, the mouthwashes formulated from *E. caryophyllata* and *M. piperita* were the least efficient (1 µg/ml). The mouthwashes based of essential oils of *O. gratissimum* (0.25 µg/ml), *O. basilicum* (0.25 µg/ml) and *C. citratus* (0.25 µg/ml) were most effective against *P. mirabilis* contrary to the mouthwashes formulated from *M. piperita* (1 µg/ml). Against *M. luteus*, the mouthwashes were more effective. Indeed, the minimum inhibitory concentration of mouthwashes formulated from *O. basilicum*, *C. citratus*, *C. anisata* and *Lippia multiflora* was 0.125 µg/ml while the minimum inhibitory concentration of mouthwashes based *O. gratissimum*, *E. caryophyllata* and *M. piperita* was 0.25 µg/ml. All mouthwashes were active against *Staphylococcus aureus* with a same minimum inhibitory concentration (0.25 µg/ml). This result is in agreement with those obtained by Baba-Moussa et al (2012) and who had shown a high inhibitory activity of essential oil of *Lippia multiflora* rich in 1,8-cineole against *Staphylococcus aureus*. Similarly, Oussalah (2007) has shown that 1,8-cineole is very active against *Staphylococcus aureus*. The effectiveness of mouthwashes formulated from *O. basilicum*, *C. citratus* and *C. anisata* were also very remarkable against the yeast strain *C. albicans* with a minimum inhibitory concentration of 0.125 µg/ml. Only the mouthwash of *L. multiflora* had a maximal inhibitory concentration against *C. albicans* (1 µg/ml).

Globally, the mouthwashes were more effective against Gram positive bacteria (*M. luteus* and *S. aureus*) than Gram negative bacteria (*P. aeruginosa* and *P. mirabilis*). Indeed, the architectural organization of cell wall of Gram positive bacteria is less complex than that of Gram negative bacteria. This complexity lies in the fact that the external membrane of Gram negative bacteria is hydrophilic and can block the penetration of hydrophobic compounds in the cell membrane (Ouattara et al., 2008). This structural difference disposes the Gram-positive bacteria more sensitive to essential oils (Kalemba and Kunicka, 2003). The sensitivity of Gram positive bacteria against the essential oils compounds was observed by other authors (Remmal et al., 1995; Deena, 2000, Kalemba and Kunicka 2003).

M. luteus used in this study was the strain more sensitive to all mouthwashes while *P. aeruginosa* strain was more resistant to these mouthwashes. The mouthwashes formulated from essential oils of *O. basilicum* and *C. citratus* were the most effective of all studied micro-organisms. This effectiveness of different mouthwashes is in agreement with effectiveness of essential oils which they derived (Bonou et al., 2016a, c).

Bactericidal profiles of different mouthwashes

Figure 2 shows the bactericidal profiles of studied mouthwashes against the five reference germs. The minimum bactericidal concentrations have varied both according to the type of mouthwash and the type of germ. It was varied from 0.125 to 1 µg/ml. With few exceptions, the effectiveness tendency observed with minimum bactericidal concentrations was the same for minimum inhibitory concentrations. We conclude that the most inhibitory mouthwashes are the most bactericidal. So the mouthwashes formulated from essential oils of *O. basilicum* and *C. citratus* are most bactericidal against all germs. Note that all mouthwashes have a bactericidal effect against the germs. These results confirm the uses of these medicinal plants by Beninese population to treat several diseases including oral diseases. Girard (2010) had reported the use of *C. anisata*, *L. multiflora*, *E. caryophyllata* and *M. piperita* in the treatment of oral diseases.

Relationship between antimicrobial activity of mouthwashes and chemical composition of essential oils

In the present study, all mouthwashes have shown inhibitory and bactericidal effects against all studied germs. The effectiveness of the mouthwashes has varied according to the mouthwashes and germs. The antimicrobial activity of each mouthwash has proven to be similar to the activity of the components of the essential oils used to formulate the mouthwashes. These differences could be explained by the fact that the essential oils used to formulate these mouthwashes not have the same chemical composition. Thus, the major components of essential oils used to formulate the two most powerful mouthwashes (Ob and Cc) are estragole (85.92%) for *O. basilicum* and neral (33.53%) and geranial (43.10%) for *C. citratus* (Table 1).

Dongmo et al (2002) found that the major compounds of most active essential oils extracted from citrus were neral and geranial. Two other mouthwashes (Og and Ca) were very active against the germs. The major compounds of essential oils used to formulate these previous mouthwashes are thymol (30.62%), para-cymene (25.25%) and gamma-terpinolene (24.24%) for *O. gratissimum* and estragole (97.10%) for *C. anisata*. Eugenol (85.90%) is the major chemical compounds of *E. caryophyllata*.

The importance of thymol in antibacterial activity of essential oil of *O. gratissimum* was shown by Oussou et al. (2010). Indeed, testing the split essential oil of *O. gratissimum* against some enterobacteria: its was seen by the authors that the mainly fraction containing oxygenated compounds principally thymol, was more active than other fractions. Indeed, thymol (family of

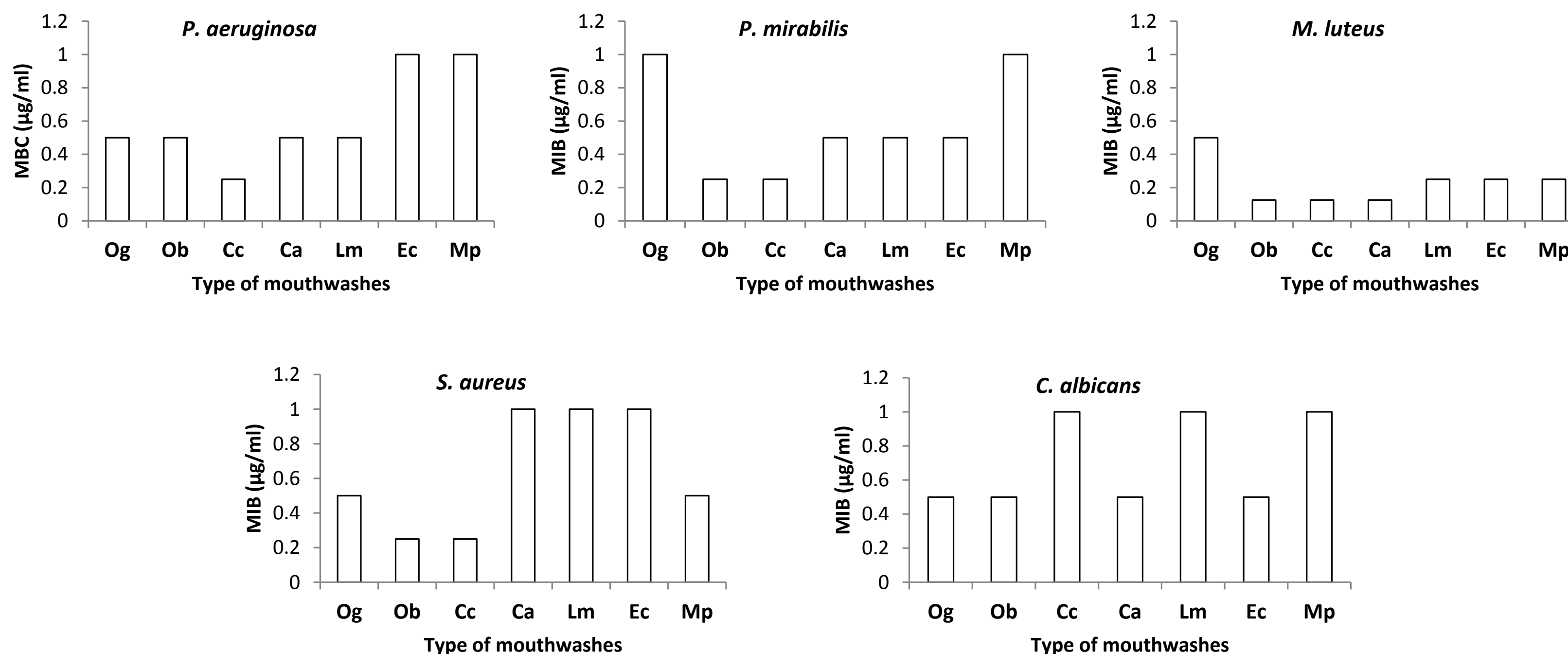


Figure 2. Minimum Bactericidal Concentration (MBC) of different mouthwashes against some reference strains. *P. aeruginosa = Pseudomonas aeruginosa; P. Mirabilis = Proteus mirabilis ; M. Luteus = Micrococcus luteus; S. Aureus = Staphylococcus aureus; C. Albicans = Candida albicans.* Og = Mouthwash formulated from *Ocimum gratissimum*; Ob = Mouthwash formulated from *Ocimum basilicum*; Cc = Mouthwash formulated from *Cymbopogon citratus*; Ca = Mouthwash formulated from *Clausena anisata*; Lm = Mouthwash formulated from *Lippia multiflora*; Ec = Mouthwash formulated from *Eugenia caryophyllata* and Mp = Mouthwash formulated from *Mentha piperita*.

monoterpene phenols) is an aromatic compound particularly present in thyme. It uses to formulate several drugs through its antibacterial, antiseptic and antifungal properties. The thymol is found to treat mouth ulcers, throat irritations and insect bites. Eugenol among to family of propenylphenols, it is very commonly used for its antiseptic and analgesic properties and used to formulate the mouthwashes and gingival pastes against oral diseases. It can also be found in ointments used to decongest the airways in case of cold or bronchitis. The estragole (methyl chavicol) belong to phenol methyl ether family is used in pharmacology for their intestinal anti-spasmodic, antibacterial and antifungal properties. Geranial has calming virtues (nervous system and muscles) and sedative properties.

On the other hand, it has antiseptic and anti-viral potentials. Inducer of the glutathione-S-transferase enzyme, geranial induce apoptosis and inhibit the promotion of skin cancers (Kapur et al., 2016).

Conclusion

The formulated mouthwashes were effective against all oral tested microorganisms. The antimicrobial activity of each mouthwash stems from the constituents of the plant essence of which it is composed. The mouthwashes formulated from essential oils of *O. basilicum* and *C. citratus* were most bactericide against the germs. They are followed in order of effectiveness to the mouthwashes formulated from essential oils

of *O. gratissimum* and *C. anisata*.

Conflict of interests

The authors have not declared any conflict of interests.

REFERENCES

Ameziane R (2014). Guide de promotion de la santé bucco-dentaire, Faculté de médecine dentaire, Rabat au Maroc. pp. 4-6.

Baba-Moussa F, Adjanohoun A, Adéoti K, Gbénou JD, Aloukoutou D, Kpavodé L, Moudachirou M, Akpagana K, Bouchet P, Kotchoni SO, Toukourou F, Baba-Moussa L (2012). Antimicrobial properties and phytochemical profiling of essential oils extracted from traditonally used medicinal plants in Benin. Int. J. Nat. Prod. Sci. 2:1-11.

Bonou J, Ahouandjinou H, Baba-Moussa F, Adéoti Z, Dougnon V, Métongnon I, Gbénou JD, Toukourou F, Baba-Moussa L (2016b). Assessment of the antimicrobial activity of essential oils from some Beninese medicinal plants: Influence of different tweens. Issues Biol. Sci. Pharm. Res. 4:43-49.

Bonou J, Baba-Moussa F, Adéoti Z, Ahouandjinou H, Dougnon V, Dossa D, Gbénou JD, Toukourou F, Baba-Moussa L (2016c). Antimicrobial activity of essential oils of *Lippia multiflora*, *Eugenia caryophyllata*, *Mentha Piperita* and *Zingiber officinale* on five oral-dental microorganisms. J. Pharmacogn. Phytochem. 5:471-476.

Bonou J, Baba-Moussa F, Noumavo PA, Ahouandjinou H, Adéoti K, Métognon I, Akpagana K, Mansourou M, Gbénou JD, Baba-Moussa L (2016a). Composition chimique et influence de différents tweens sur le pouvoir antimicrobien des huiles essentielles de *Ocimum gratissimum*, *Ocimum basilicum*, *Laurus nobilis* et *Melaleuca quinquenervia*. Euro. Sci. J. 12:1857-7881.

Deena MJ, Thoppil JE (2000). Antimicrobial activity of the essential oil of *Lantana camara*. Fitoterapia 71:453-455.

Dongmo PM, Boyom FF, Ducelier D (2002). Chemical composition and *in vitro* antifungal activity of essential oils of Citrus on mycelial growth of *Phaeoramularia angolensis*. J. Books Serv. Comp. EDP. 1:95-104.

Farshori NN, Al-Oqail MM, Al-Sheddi ES, Siddiqui MA, Rauf A (2013). Antimicrobial potentiality of *Polyalthia longifolia* seed oil against multi drug resistant (MDR) strains of bacteria and fungus of clinical origin. Afr. J. Microbiol. Res. 19:1977-1982.

Girard G (2010). Les propriétés des huiles essentielles dans les soins bucco-dentaires d'hier et aujourd'hui : mise au point d'un modèle préclinique de lésion buccale de type aphte pour tester les effets thérapeutiques des huiles essentielles, thèse de Pharmacie, Université de Henri Poincare-Nancy1, France. pp. 45-53.

Kapur A, Felder M, Fass L, Kaur J, Czarnecki A, Rathi K, Zeng S, Osowski KK, Howell C, Xiong MP, Whelan RJ, Patankar MS (2016). Modulation of oxidative stress and subsequent induction of apoptosis and endoplasmic reticulum stress allows citral to decrease cancer cell proliferation. Sci. Rep. 6:27530, DOI: 10.1038/srep27530

Osséni AML, Agbangnan DCP, Bossou A, Yédomonhan HP, Avlessi F, Sohounhloué KCD (2014). Radical scavenging activities and study of volatile compounds of three plants used in traditional medicine in Benin: *Anchomanes difformis*, *Parkia biglobosa* and *Polyalthia longifolia*. Int. J. Innov. Appl. Stud. 9:1609-1619.

Ouattara S, Kra MKA, Kporou EK, Guede-Guina F (2008). Evaluation de l'activité antifongique des extraits de *terminalia ivorensis* (tekam2) sur la croissance *in vitro* de *Candida albicans.* Rev. Ivoir. Sci. Technol. 12:205-214.

Oussalah M. Caillet S, Saucier L, Lacroix M (2007). Inhibitory effects of selected plant essential oils on the growth of four pathogenic bacteria: *E. coli* 0157:H7, *Salmonella Thyphimurium*, *Staphylococcus aureus* and *Listeria monocytogenes*. Food Control. 18:414-420.

Oussou KR, Yolou SF, Tue Bi B, Kanko C, Boti JB, Ahibo C, Casanova J (2010). Etude Chimique Bio-Guidée de l'huile Essentielle de *Ocimum Gratissimum* (Lamiaceae). Euro. J. Sci. Res. 40:50-59.

Peluchonneau C. (2011). La carie dentaire : prévention et conseil à l'officine, limoges, France. P 8.

Quenum ROA, Baloîtcha Olurémi DR (2003). Contribution to the study of antimicrobial properties of extracts of *Lantana camara L.* and *Hyptis suaveolensis* Poit *Ocimum gratissimum* L. on five coetaneous germs tropism. Dissertation study of Master, University of Abomey-Calavi, Benin.

Remmal A, Bouchikhi T, Rhayour K, Ettayebi M, Tantaoui-Elaraki A (1993). Improved method for determination of antimicrobial activity of essential oils in agar medium. J. Ess. Oil. Res. 5:179-184.

Sekoussounon S (2012). Evaluation de la qualité microbiologique des phytomédicaments améliorés vendus dans les officines de pharmacie de la ville de Cotonou (Bénin). Thèse de pharmacie, Université d'Abomey Calavi, Bénin.

Wheater PR, Young B, Heath JW (2004). Histologie fonctionnelle. Bruxelles, De Boeck Supérieur. P 424.

Yayi-Ladekan E, Kpoviessi DSS, Gbaguidi F, Kpadonou-Kpoviessi BGH, Gbénou J, Jolivalt C, Moudachirou M, Accrombessi GC, Quetin-Leclercq J (2011). Variation diurne de la composition chimique et influence sur les propriétés antimicrobiennes de l'huile essentielle de *Ocimum canum* Sims cultivé au Bénin. Int. J. Biol. Chem. Sci. 5:1462-1475.

Yee R, Sheiham A. (2002). The burden of restorative dental treatment for children in third world countries. Int. Dent. J. 52:1-9.

academicJournals

Vol. 10(43), pp. 1813-1821, 21 November, 2016
DOI: 10.5897/AJMR2016.8105
Article Number: 923355061661
ISSN 1996-0808
Copyright © 2016
Author(s) retain the copyright of this article
http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Full Length Research Paper

Characterization of arbuscular mycorrhizal fungi in apple (*Malus domestica* Borkh) growing area in Kashmir Himalaya (India): A case study of Bandipora district

Misbah A. Malik*, Zargar M. Y. and Baba Z. A.

Faculty of Agriculture, Sher e Kashmir University of Agricultural Sciences and Technology of Kashmir-Wadoora, Sopore-193201, India.

Received 12 May, 2016; Accepted 5 July, 2016

Rhizosphere soil samples and root pieces of 3 year old apple trees (var. Red delicious) were collected from 10 villages of district Bandipora of Kashmir Himalaya. Samples were processed for isolation of arbuscular mycorrhizal spores and their identification. On the basis of various morphological characters such as spore shape, colour, size, hyphal colour and with the help of International Collection of Vesicular-Arbuscular Mycorrhizal (INVAM), the isolated genera were identified as *Acaulospora, Scutellospora*, *Gigaspora,* and *Glomus*. *Glomus* spores were more predominant in the district followed by *Acaulospora*. Spores of *Gigaspora* were larger in diameter while as *Glomus* spores were smaller. *Glomus* species showed higher root colonization from Markondal followed by *Acaulospora* species from Gorura, *Gigaspora* species from Ajas and *Scutellospora* species from Sangri. The highest biological activity was due to adequate application of organic manures in the soil and also due to application of fertilizers.

Key words: Arbuscular mycorrhizal fungi (AMF), apple, *Acaulospora*, *Scutellospora*, *Gigaspora*, *Glomus*, root colonization, Kashmir.

INTRODUCTION

Apple (*Malus domestica* Borkh) is considered as one of the most important and widely grown fruits in temperate zones of the world with regard to its acreage, production, economic returns and high nutritive value. Apple is the fourth widely produced fruit in the world after banana, orange and grapes. Indian apple production averages nearly 1.4 million making it the sixth largest apple producer in the world (Satish et al., 2006) and second in Asia (Deepa, 2008). In India, apple is mainly grown in three mountainous states of North India, viz. Himachal Pradesh, Jammu and Kashmir and Uttarakhand where they are typically grown at an altitude of 4000 to 11000 ft.

*Corresponding author. E-mail: misbamalik98@gmail.com.

Author(s) agree that this article remains permanently open access under the terms of the Creative Commons Attribution License 4.0 International License

Jammu and Kashmir and Himachal Pradesh have almost equal area under apple, but later has the highest average yield and accounts for 67% of the total apple production (Masoodi, 2003) and 50% of its export in the country, earning a substantial foreign exchange. Productivity is much higher than national average of 6.86 tons/ha. India annually exports apple worth Rs. 400 million (Nearly US $ 10 million) out of which apples worth Rs. 200 million come from Kashmir. Moreover, it provides job opportunity to 1.2 million people directly or indirectly. In horticulture sector, the largest area of 43.53% is occupied by apple out of total area under fruit and 65.46% out of fresh fruit area (Anonymous, 2007), thereby making it the largest contributor to the state GDP among the horticulture produce.

Bandipora is a newly carved district from erstwhile Baramulla district. The district is surrounded by Himalayan Mountains having district Kargil on north, Kupwara in west, Baramulla in south and Ganderbal in east. The district is situated between 34°25´ and 34°42´ N Latitude and 74°39´ and 74°65´ E longitude. It has unique agro climatic conditions of low temperature even during summer months. The main fruit crop of the district is apple. Out of the total area of 140156 hactare under apple in Kashmir district Bandipora covers area of 5605 hactare (Anonymous, 2013). Bandipora is known for the better quality apple in the valley.

Phosphorus which is the second primary nutrient after nitrogen has a direct effect on yield and tree health. It is also important in determining fruit size, firmness, colour and storage potential. It increases the potential of fruiting by increasing the number of flower clusters, their intensity and the level of fruit set. But, phosphorus is an extremely immobile element in soils and even if it was added to soil in soluble form, it becomes immobilized as organic phosphorus, calcium phosphates, or other fixed forms (Jackson and Mason, 1984). AM-fungi are known to be effective in increasing phosphorus uptake in many crops, particularly in low phosphorus soils (Osonubi et al., 1991). They are associated with improved growth of many plant species due to increased nutrient uptake, production of growth promoting substances, tolerance to drought and synergistic interaction with other beneficial soil micro-organisms such as N-fixers and P-solubilizers (Sreenivasa and Bagyaraj, 1989). Arbuscular mycorrhizal fungi play an important role in sustainable agriculture as well as agricultural ecosystem management. The important genera of endomycorrhizal fungi reported so far are *Acaulospora, Entrophosphora, Gigaspora, Glomus, Sclerocystis* and *Scutellospora* (Morton, 1988). All these fungi are obligately associated with plant roots and develop symbiotic relationship with their hosts. Different species can be differentiated on the basis of sporocarp size, spore dimension, presence/absence of hyphal mantles, spore ornamentation (warts, wrinkles, pits, reticulum, spines, etc.), spore walls, spore content, hyphal attachment, manner of spore germination, histochemical reaction, etc. (Schenck and Smith, 1982). Keeping in view the importance of arbuscular mycorrhizal fungi in phosphorus solubilization and very little work having been done on arbuscular mycorrhizae with respect to apple, the present investigation was taken up with the objective of isolation and purification of arbuscular mycorrhizal spores from the rhizospheric soil of apple, morphological characterization of the spores upto generic level and their root colonization studies.

MATERIALS AND METHODS

Study area

Bandipora is situated on the banks of the Wular, the largest fresh-water lake in Asia, with geographic coordinates of 34.5052° N, 74.6869° E. In Bandipora district, apple production has swelled to 69,147 MT in 2014-15 from 65,102 MT in 2013-14. Similarly, the area under crop extended from 5,605 to 5,840 hectares in one year and thus contributes a lot in horticulture sector of Jammu and Kashmir. Ten villages (Arigam, Ajas, Asham, Gorura, Nadihal, Naidkhai, Sumbal, Markondal, Sangri, Watlab) were selected from district Bandipora. From each village three orchards were randomly chosen and from each orchard five rhizosphere soil samples were drawn which were composited into one representative sample. Most of the orchardists followed the pesticide schedule as per Department of Horticulture, Government of Jammu and Kashmir (Anonymous, 2015).

Isolation and purification of arbuscular mycorrhizal spores

Rhizosphere soil samples were collected from the feeder roots on all sides of the canopy of the tree (fruiting stage). The soil samples taken from the rhizosphere of apparently healthy apple trees were collected in June 2014. The soil samples (about 250 g each) were immediately brought to the laboratory, air-dried and processed for AM isolation using wet sieving and decantation method (Gerdemann and Nicolson, 1963). Counting of spores was done under microscope Olympus CH20i with magnification of 10×40.

Measurement of available sulphur, soil organic carbon, available phosphorus, available potassium

Available sulphur, soil organic carbon, available phosphorus, available potassium were studied as per Chesnin and Yein (1951), Walkley and Black (1934), Olsen et al. (1954), and Stanford and English (1949), respectively. Soil used during the present study contained 1.74% organic carbon, 357.43 kg/ha available nitrogen, 17.05 kg/ha phosphorus, 12.02 kg/ha available sulphur and 185.38 kg/ha available potassium and all these were in medium range.

Identification

The arbuscular mycorrhizal fungal species were morphologically identified on the basis of spore size, spore colour and spore wall up to the genera level as per guidelines by INVAM (http://invam.caf.wvu.edu).

Root colonization studies of the isolated spores

The isolated spores were further purified and mass multiplied on

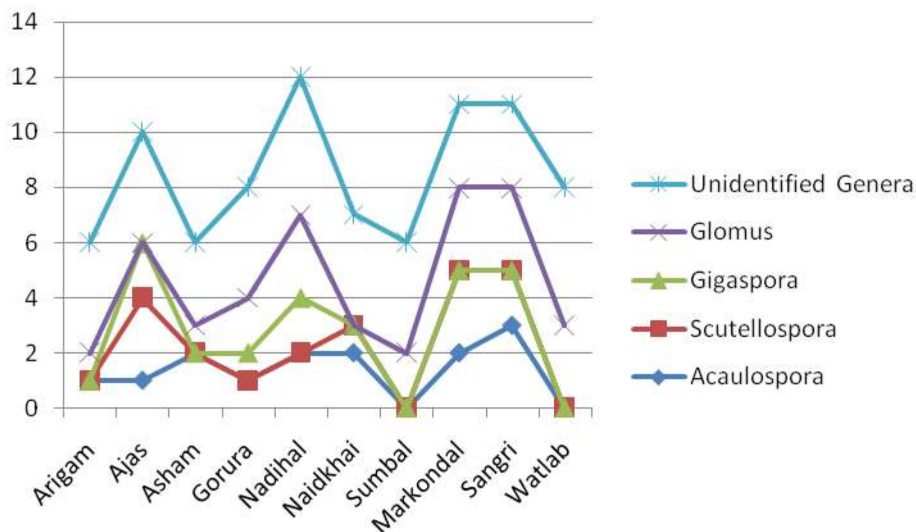


Figure 1. Isolation of arbuscular mycorrhizal spores from rhizospheric soil of apple from different locations of district Bandipora.

maize. Surface sterilized healthy maize seeds, pre-germinated in Petri plates under aseptic conditions, were sown in polythene bags containing sterilized soil + sand mixture (1:2 w/w). These bags were aseptically inoculated with identical AM spores at 5 cm depth (Jackson, 1973). The bags were kept in a greenhouse at 25±3°C and irrigated with sterile water. The plants were uprooted after 45 days. The roots were collected, washed with sterile water to remove adhering soil debris and observed for mycorrhizal infection. The infectivity was proved by noticing the presence of hartig net, vesicles, asbuscules or hyphae of endophytes on roots.

For estimating mycorrhizal root colonization, the root samples were collected and washed carefully to remove the adhering debris. The tertiary roots were cut into small pieces of approximately 1 cm length and subjected to differential staining as described by Phillips and Hayman (1970). The estimation of mycorrhizal infection in roots was made by visual observation (Giovannetti and Mosse, 1980). A randomly selected aliquot of stained root segments, suspended in water, was spread in a Petridish viewed under a dissecting microscope at a magnification of 10 and 40×. In case of AM colonization, root segments containing vesicles and arbuscules of endophyte and number of mycorrhizal short roots were considered infected as suggested by Beckjord et al. (1984).

Per cent mycorrhizal infection = Number of infected root segments / Total number of segments examined × 100

The data recorded during the investigation was statistically analyzed with the help of Pearson correlation (Gomez and Gomez, 1984).

RESULTS AND DISCUSSION

Morphological characterization of arbuscular mycorrhizal spores

Spore morphology and wall characteristics were considered for the identification of arbuscular mycorrhizal fungi. The spores isolated from soils of district Bandipora were identified upto generic level using bibliographies provided by Walker et al. (2007). Four types of genera were isolated. The genera isolated were *Acaulospora, Scutellospora*, *Gigaspora,* and *Glomus*. There were 3 to 6 unidentified spores per gram from all studied locations (Figure 1).

Acaulospora spores were present singly in the soil and develop laterally on the neck of asporiferous saccule. Spores were light orange to yellowish brown (Table 1 and Figures 4, 5, 6 and 7), globose to sub-globose in shape and 150 to 210 μm in diameter. These spores were triple layered with L1 which forms the spore surface light yellow to apricot yellow in colour and 0.7 to 2.0 μm in thickness. L2 was laminate and light orange to yellowish brown, 6.8 to 7.4 μm in thickness. L3 was laminate, hyaline, 0.8 to 1.6 μm in thickness and usually tightly adherent to L2. Similar observations have been reported by others also (Walker et al., 2007; Sharma et al., 2009). *Gigaspora* spore wall consisted of a permanent outer layer enclosing a laminate layer, each with different properties that distinguish species (e.g. color, thickness, etc). Our observations corroborate with those of Koske (1987) and Bentivenga and Morton (1995). *Scutellospora* spores were with or without ornamentations. Spores consisted of a bilayered spore wall and two bilayered flexible inner walls. Thin-walled auxillary cells with smooth to knobby surfaces were produced on hyphae in the soil near the root surface and were also reported by Schenck and Perez (1990). The spore colour of the species of *Glomus* was of wide range. It varied from red-brown to almost black or straw to dark orange but most were yellow brown in colour. Spores possessed globose to sub-globose shape, about 40 to 120 μm in size. Spore wall consisted of three layers (L1, L2 and L3). Our findings corroborate with those of many other workers (Koske, 1984;

Table 1. Morphological features of isolated genera of AM fungi.

Genera	Spore size (µm diameter)	Spore shape	Spore colour	Spore wall	Hyphal colour
Acaulospora	115-170	Globose to sub globose	Yellow brown to dark brown	Three layered (L1, L2 and L3)	Grey white
Gigaspora	200-300	Globose to sub globose	White to cream usually a rose pink tint.	Bilayered layered (L1 and L2)	Orange brown
Scutellospora	100-170	Sub globose to ellipsoid to oblong	Cream to yellow or pale orange brown to dark orange brown	Bilayered spore wall (L1 and L2)	Hyaline to orange white.
Glomus	40-120	Globose to ellipsoid	Red brown to almost black most are yellow brown	Three layered (L1, L2 and L3)	Hyaline to yellowish.

Table 2. Correlation between spore population and other studied parameters of district Bandipora.

Parameter	Spore population
Spore population	1
Organic carbon	0.752*
Available nitrogen	0.626
Available phosphorus	-0.543*
Available sulphur	0.561
Available potassium	0.599
Root colonization	0.613*

*Correlation is significant at the 0.05 level.

Koske and Gemma, 1990).

There was no evidence of any ectomycorrhizal association with apple roots, and this corroborates with the findings of Greene et al. (1982). *Glomus* species was common and made up for more than 75% of total isolates followed by *Acaulospora, Gigaspora* and *Scutellospora.* Dominancy of *Glomu*s in the present study is in agreement with the findings of many other workers (Mridha and Dhar, 2007; Burni et al., 2009; Sharma et al., 2009). The predominance of *Glomus* spp. under varying soil conditions might be due to the fact that they are widely adaptable to the varied soil conditions and survive in acidic as well as in alkaline soils (Pande and Tarafdar, 2004).

Root colonization studies of arbuscular mycorrhizal fungi

In the current study, the AM colonization in the apple roots from Bandipora district varied between 62.04 and 81.13% (Figure 2, 3, 8, 9, 10 and 11). The results are in conformity with the Kandula et al., (2006) who also observed higher colonization in the apple roots and confirmed the ubiquitous nature of AMF spores. The highest root colonization was recorded in response to the inoculation with *Glomus* spp. (81.13%) followed by *Acaulospora* species (75.34%), *Gigaspora* species (73.13%) and *Scutellospora* species (70.00%). Similar results were reported by some workers (Hosamani et al., 2004; Smith and Read, 2008).

Results of the present study indicate that the nutrient contents of the soils played a significant role in occurrence of different species of arbuscular mycorrhizal fungi and it is evident from the Perusal of the data presented in Table 2 which revealed that AM spore population of district Bandipora was positively and significantly correlated with organic carbon (r=0.752*). The results are in conformity with those of Lipinski et al. (2003) who also reported a significant positive correlation between soil organic carbon and AM spore population. Negative relationship of AM spore population with available phosphorus (-0.543*) content in the soil was also reported. Stribley et al. (1980) who also observed that infection developing under conditions of high phosphorus availability may function parasitically without making any beneficial contribution to plant nutrient supply. Our findings corroborate with those of Graham and Timmer (1984) and Wu et al. (2006). There was a significant correlation between AM spore population and root colonization (r=0.613*) in district Bandipora. Kumar et al. (2013) also found a significant positive correlation between mycorrhizal spores and colonization. Yang et al. (2010) found a positive correlation between and mycorrhizal colonization and spores. These results are also supported by Li et al. (2009). Since the climatic conditions of the study area fall under temperate zone which are conducive to the mycorrhizal development, it is possible that concentration of such propagules may be higher (Akhter, 2005).

Moreover, influence of apple roots through their exudates cannot be ruled out which needs further studies.

Conflicts of interests

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENTS

The authors are thankful to Sher e Kashmir

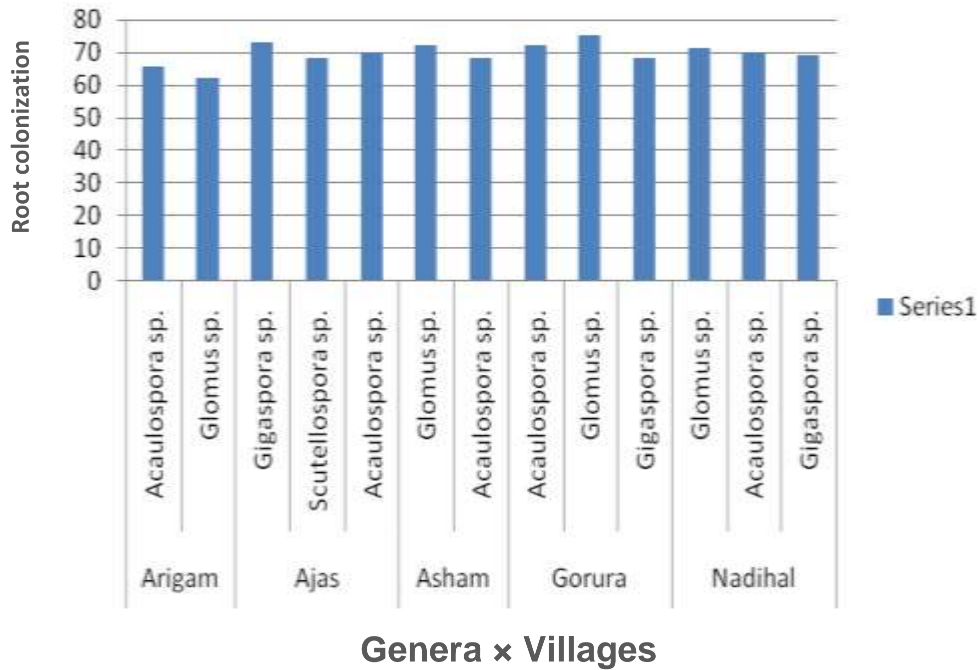


Figure 2. *In-vitro* root colonization by AM fungal spores isolated from district Bandipora.

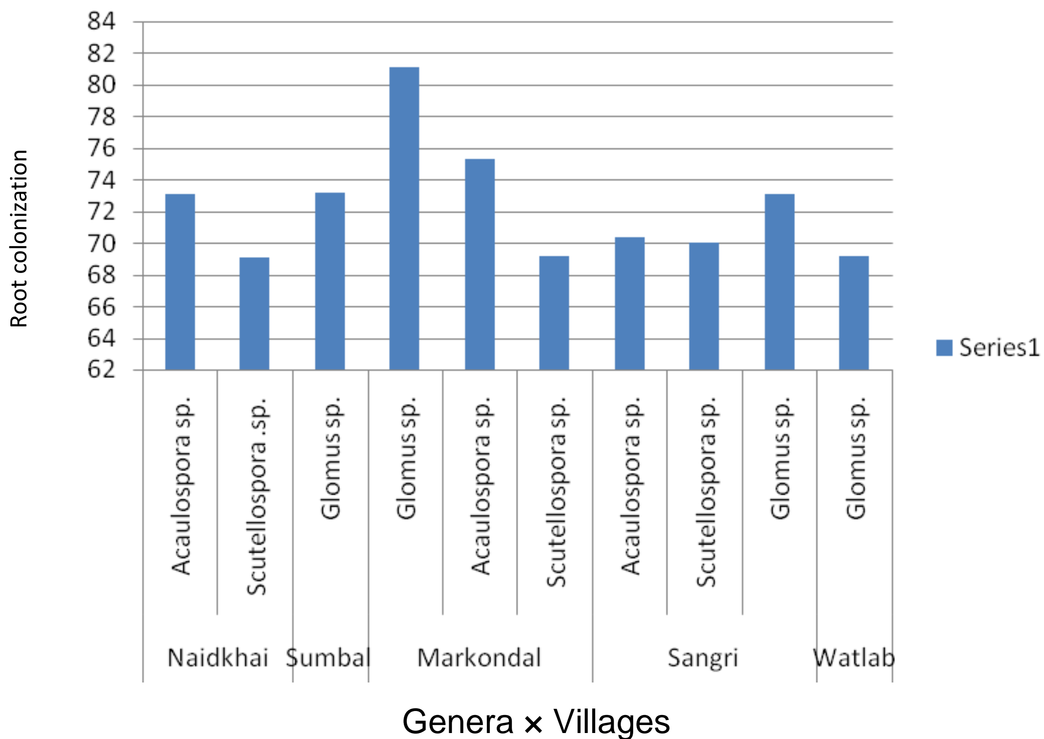
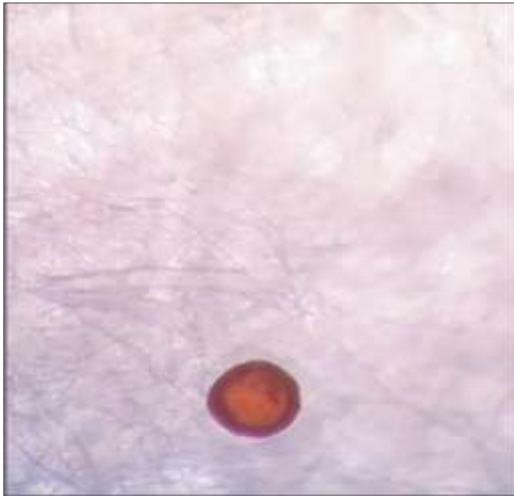


Figure 3. *In-vitro* root colonization by AM fungal spores isolated from district Bandipora.

University of Agricultural Science and Technology of Kashmir for providing laboratory facilities; the faculty for rendering there kind help and support; and help rendered by the orchardists during collection-cum-surveys trips is



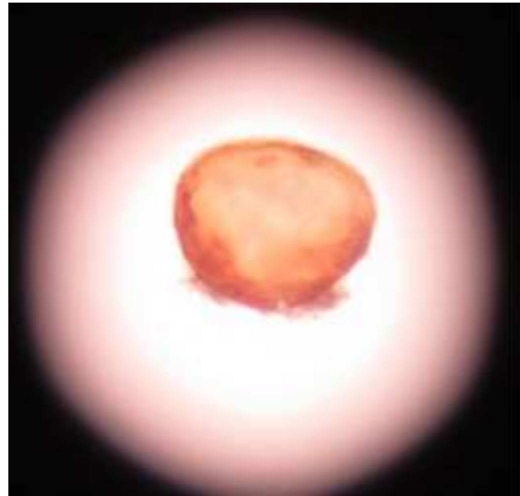
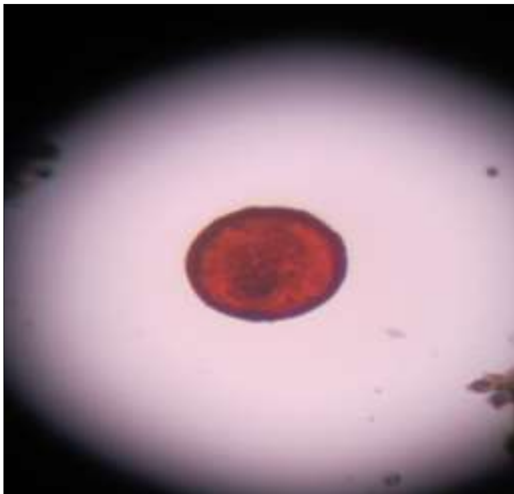


Figure 4. Spores of the genus *Acaulospora*.



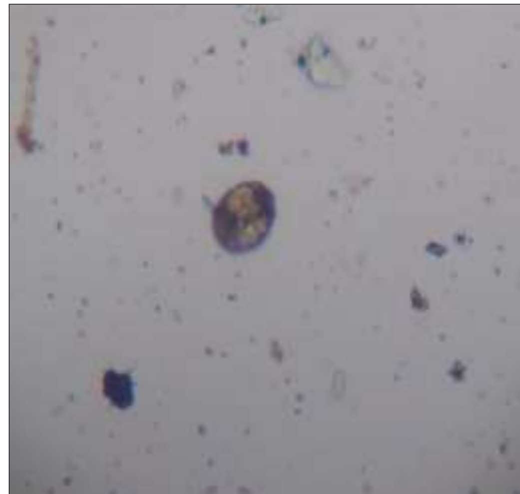


Figure 5. Spores of the genus *Glomus*.

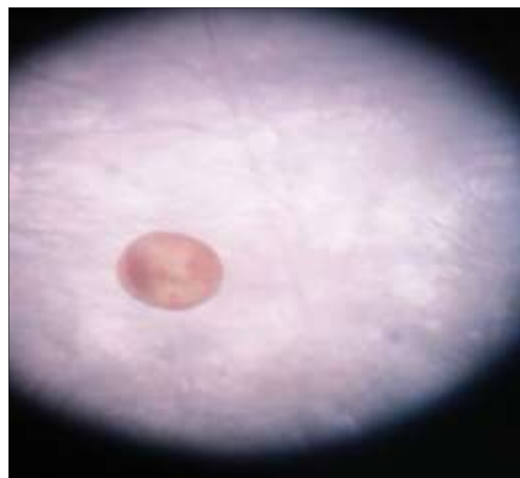


Figure 6. Spores of the genus *Gigaspora*.

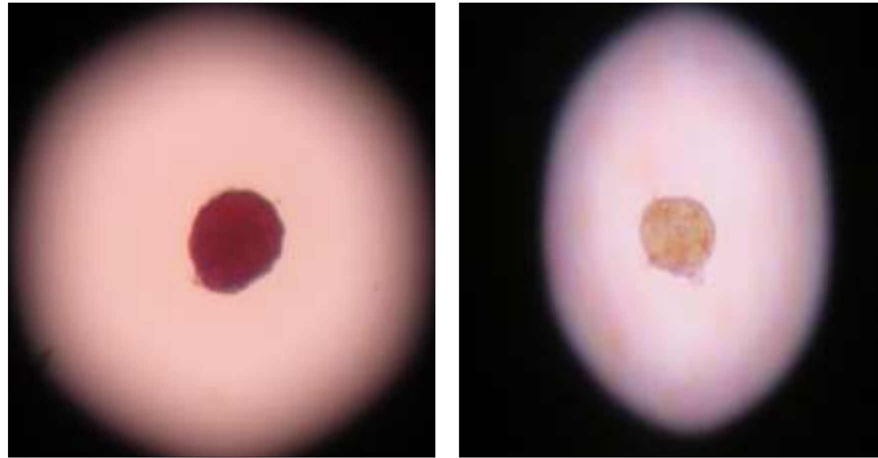


Figure 7. Spores of the genus *Scutellospora*.

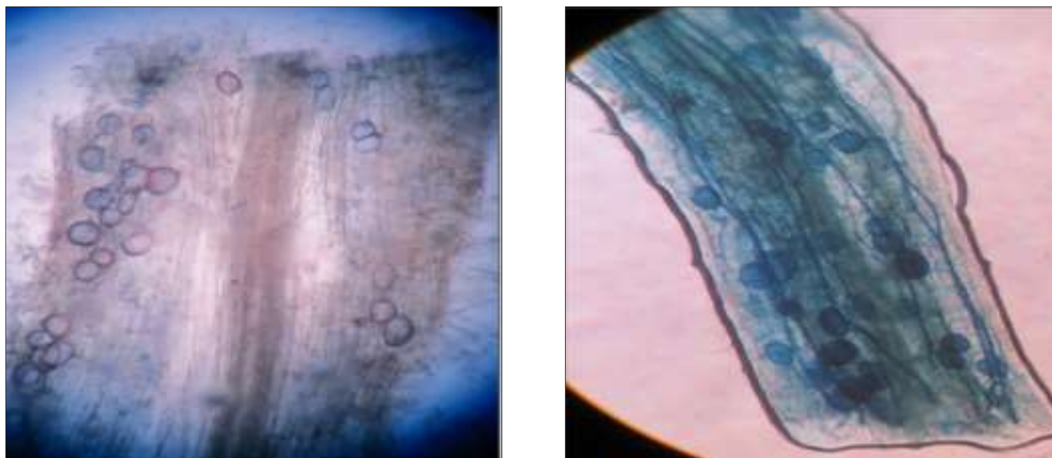


Figure 8. Root colonisation of the genus *Acaulospora*.

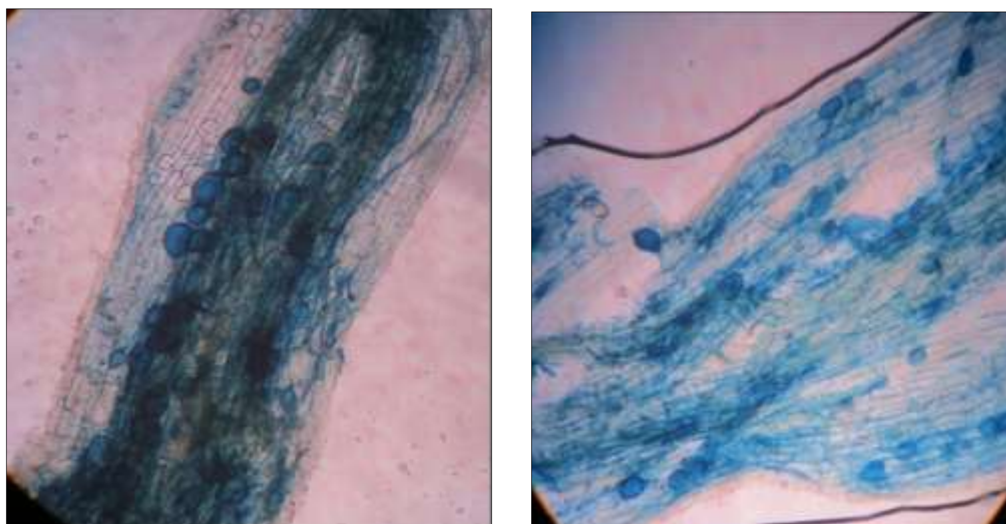


Figure 9. Root colonisation of the genus *Scutellospora*.

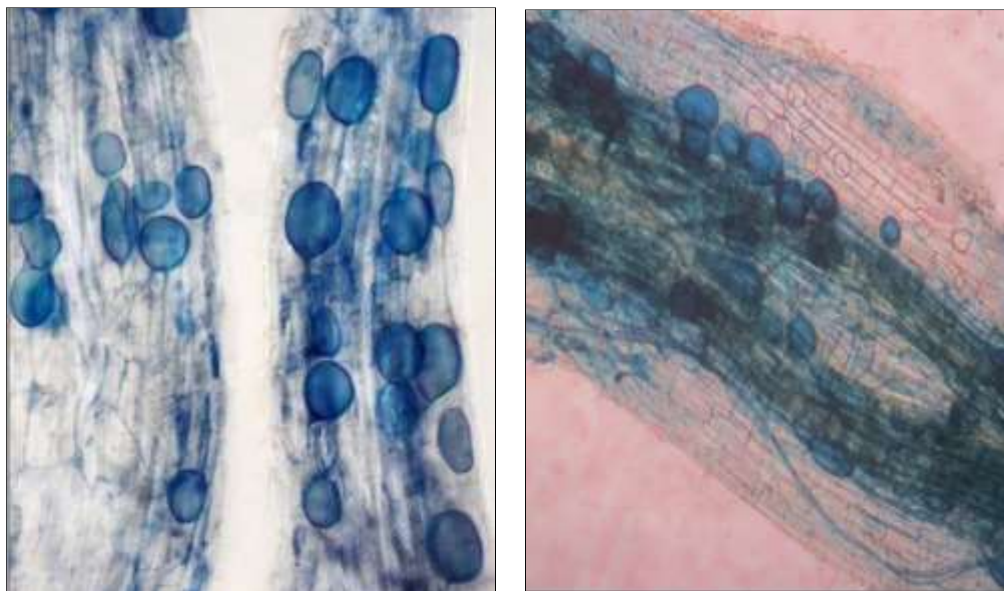


Figure 10. Root colonisation of the genus *Glomus*.

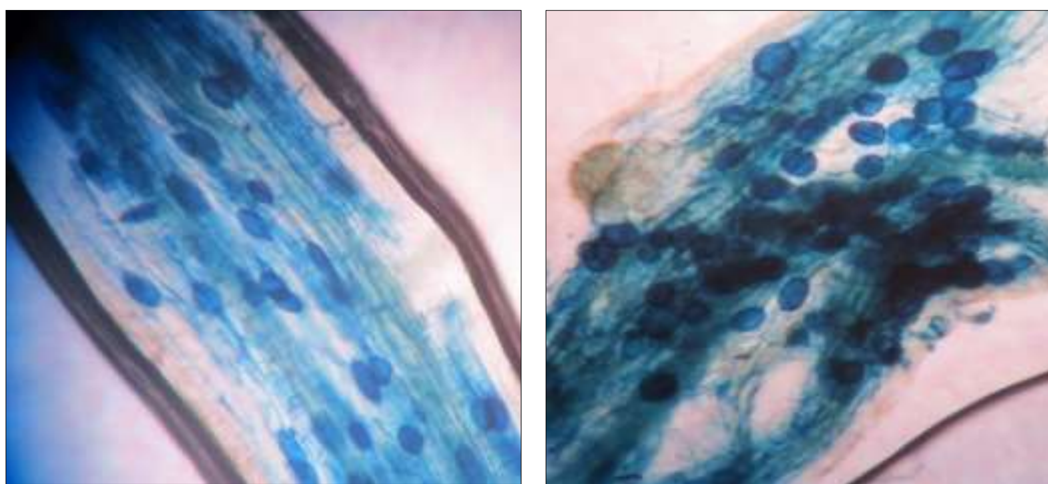


Figure 11. Root colonisation of the genus *Gigaspora*.

highly put on record.

REFERENCES

Akhter F (2005). Studies on relationship between fruit yield and quality with soil and leaf nutrient content in apple orchards of Zangier block of district Baramulla Kashmir. University of Kashmir Digital Repository.

Anonymous (2007). Area and Production of main Fruits in Jammu and Kashmir State. Department of Horticulture, Government of Jammu and Kashmir, Srinagar, J&K.

Anonymous (2013). Area and Production of main Fruits in Jammu and Kashmir State. Department of Horticulture, Government of Jammu and Kashmir, Srinagar, J&K.

Anonymous (2015).Area and Production of main Fruits in Jammu and Kashmir State. Department of Horticulture, Government of Jammu and Kashmir, Srinagar, J&K.

Beckjord PR, McIntosh MS, Hacskaylo E, Melhuish JH (1984). Inoculation of Loblolly Pine Seedlings at Planting with Basidiospores of Ectomycorrhizal Fungi in Chip Form. Research Note No. 324, Forest Service, North-Eastern Forest Experiment Station, Florida, USA, P 25.

Bentivenga SP, Morton JB (1995). A monograph of the genus Gigaspora, incorporating developmental patterns of morphological characters. Mycologia 87:720-732.

Burni T, Iftikhar S, Jabeen M, Zainab SB (2009). Diversity of VA (Vesicular Arbuscular) fungi in some weeds of cauliflower fields of Peshawar, Pakistan. Pak. J. Plant Sci. 15(1):59-67.

Chesnin L, Yien CH (1951). Turbidimetric determination of available sulphur. Proc. Soil Sci. Soc. Am. 15:149-151.

Deepa D (2008) Indian product, “Brief India fresh fruit sector”, Holy Higgins US Embassy.

Gerdemann JW, Nicolson TW (1963). Spores of mycorrhizal Endogone species extracted from soil by wet sieving and decanting method. Trans. Br. Mycol. Soc. 46:235-245.

Giovannetti M, Mosse B (1980). An evaluation of techniques for measuring vesicular arbuscular mycorrhizal infection in roots. New Phytol. 84:489-509.

Gomez KA, Gomez AA (1984). Statistical procedure for Agricultural Research An international Rice Research Institute Book. John Wiley and sons, 2nd edition 190.

Graham JH, Timmer LW (1984). Vesicular-arbuscular mycorrhizal development and growth response of rough lemon in soil and soilless media: Effects of phosphorus sources. J. Am. Soc. Hortic. Sci. 109:118-121.

Greene DW, Manning WJ, Cooley DR (1982) Effect of the ectomycorrhizal fungus Pisolithustinctorius and auxin rooting formulations on growth of 'Cortland' apple trees. Hortic. Sci. 17:655-656.

Hosamani PA, Lakshman HC, Kadam LB, Kiran P (2004) Screening of Arbuscular mycorrhizal fungi and their comparative efficacy on Withaniasomnifera Dunl. Nat. Environ. Pollut. Technol. 3(2):201-204.

INVAM website (2005). http://invam.caf.wvu.Edu/fungi/taxonomy/classification.html.

Jackson ML (1973) Soil Chemical Analysis. Prentice Hall India, New Delhi, India, P 498.

Jackson RM, Mason PA (1984). Mycorrhiza. Edward Arnold, Ltd., London: 60. ISBN 0-7131- 2876-3.

Kandula K, Jones E, Stewart A, Horner J (2006). Colonisaton of apple roots by arbuscular mycorrhiza in specific apple replant disease affected soil. New Zealand Plant Prot. 59:92-96.

Koske RE (1984). Spores of VAM fungi inside spores of VAM fungi. Mycologia 76:853-862

Koske RE (1987). Distribution of VA mycorrhizal fungi along a latitudinal temperature gradient. Mycologia 79:55-68.

Koske RE, Gemma JN (1990) VA mycorrhizae in strand vegetation of Hawaii: evidence for long-distance codispersal of plants and fungi. Am. J. Bot. 77:466-474.

Kumar S, Chaudhuri S, Maiti SK (2013). Soil dehydrogenase enzyme activity, in Natural and mine soil - a review. Middle-East J. Sci. Res. 13(7):898-906.

Li L, Li T, Zhang Y, Zhao Z (2009). Molecular diversity of arbuscular mycorrhizal fungi and their distribution patterns related to host-plants and habitats in a hot and arid ecosystem, southwest China. FEMS Microbiol. Ecol. 71:418-427.

Lipinski W, Terelak H, Motowicka T (2003). Suggestion for liming values of sulphatesulphur content in mineral soils for fertilization advisory needs. Soil Sci. Annu. 54(3):79-84.

Masoodi MA (2003). Agriculture in Jammu and Kashmir a perspective. Mohisarw Book Series Srinagar P 195.

Morton JB (1988). Taxonomy of VAM fungi, classification, nomenclature and identification. Mycotaxon 32:267-324.

Mridha M, Dhar P (2007). Biodiversity of Arbuscular Mycorrhizal colonization and spore population in different Agroforestry trees and crop species growing in Dianajpur Bangladesh. J. Forest. Res. 18(2):91-96.

Olsen SR, Cole CV, Watanabe FS, Dean LA (1954). Estimation of available phosphorus in soils by extraction with sodium bicarbonate. U.S. Dept. Agric. Circ. P 939.

Osonubi O, Mulongoy K, Awotoye O, Atayese M, Okali U (1991). Effects of ectomycorrhizal and vesicular-arbuscular mycorrhizal fungi on drought tolerance of four leguminous woody seedlings. Plant Soil 136:131-143.

Pande M, Tarafdar JF (2004) Arbuscular Mycorrhizal fungal diversity in Neem based Agroforestry Systems in Rajistan. Appl. Soil Ecol. 26:233-241.

Phillips JM, Hayman DS (1970) Improved procedures for clearing roots and staining parasitic and vesicular arbuscular mycorrhizal fungi for rapid assessment of infection. Trans. Br. Mycol. Soc. 55:150-161.

Satish YD, Maurice L, Barry K (2006) Prospects for India's Emerging Apple Market, USDA 8.

Schenck NC, Parez Y (1990). Manual for the identification of VA mycorrhizal fungi INVAM, University of Florida, Gainesville USA 1-283.

Schenck NC, Smith GS (1982). Additional new and unreported species of mycorrhizal fungi (Endogonaceae) from Florida. Mycologia 77:566-574.

Sharma D, Kapoor R, Bhaytnagar AR (2009). Differential growth response of Curculigoorchoides to native AMF communities varying in number and fungal components. Euro. J. Soil Biol. 45(4):328-333.

Smith SE, Read DJ (2008). Mycorrhizal Symbioses. Academic Press, London, U.K. Assessment of Mycorrhizal Frequency in the Roots of Fruit Plants Using Different Dyes.

Sreenivasa MN, Bagyaraj DJ (1989). Use of pesticide for mass production of vesicular-arbuscular mycorrhizal inoculum. Plant Soil 119:127-132.

Stanford S, English (1949). Use of the flame photometer in rapid soil tests of potassium and calcium. Agron. J. 41:446-447.

Stribley DP, Tinker PB, Rayner JH (1980). Relation of internal phosphorus concentration and plant weight in plants infected by vesicular arbuscular mycorrhizae. New Phytol. 86:261-266.

Walker C, Vestberg M, Demircik F, Stockinger H, Saito M, Nishmura I, Schubler A (2007). Molecular phylogeny and new taxa in the *Archaesporales* (*Gloremycota*): *Ambiospora feennicagen sp*.nov., *Ambisporacea*e fam nov., and emendation of *archaeospora* and *Archaesporaceae*. Mycol. Res.111:137-153.

Walkley A, Black IA (1934). An examination of the Degtjareff method for determining soil organic matter and a proposed modification of the chromic acid titration method. Soil Sci. 37:29-38.

Wu Q, Xia R, Zou Y (2006). Arbuscular mycorrhizal fungal growth on citrus roots and its correlations with soil available phosphorus content and phosphatase activity. Ying Yong Sheng Tai Xue Bao 17:685-690.

Yang RY, Zan ST, Tang JJ, Chen X, Zhang Q (2010) Variation in community structure of arbuscular mycorrhizal fungi associated with a Cu tolerant plant- *Elsholtzia splendens*. Appl. Soil Ecol. 44:191-197.

academicJournals

Vol. 10(43), pp. 1822-1828, 21 November, 2016
DOI: 10.5897/AJMR2016.8268
Article Number: E88D5C761663
ISSN 1996-0808
Copyright © 2016
Author(s) retain the copyright of this article
http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Full Length Research Paper

Storage behaviour and functionality of a heat stabilized starter derived from an African opaque sorghum beer

Djegui K. Y., A P. P. Kayodé* and J. D. Hounhouigan

Ecole de Nutrition et des Sciences et technologies Alimentaires, Faculté des Sciences Agronomiques, Université d'Abomey-Calavi, 01 BP 526 Cotonou, Bénin.

Received 13 August 2016, Accepted 13 October, 2016

Tchoukoutou is a traditional sorghum opaque beer produced and consumed in Benin. It is obtained from an uncontrolled fermentation of sorghum wort using a traditional starter called "*kpete-kpete*". The present study assessed the effects of storage duration on the physicochemical and microbiological characteristics as well as on the functional properties of the heat stabilized starter produced from "*kpete-kpete*". The physicochemical characteristics of the stabilized starter were not significantly affected by the storage duration. After 60.0 days of storage, the dry matter, pH and titratable acidity were stable with mean values of 92.5%, 4.76 and 1.33 g/kg, respectively. However, the microbial characteristics of the product were significantly affected by the storage duration. Yeasts, lactic acid bacteria (LAB) and total mesophilic aerobic counts were modified following three phases: a latency phase (between 0 and 10 days), an exponential growth phase (between 10 and 40 days) and the decline phase (between 40 and 60 days). As compared to the stabilized starter, the physicochemical characteristics of "*kpete-kpete*" were extensively modified after 15 days of storage. In the traditional "*kpete-kpete*", all the major groups of microorganisms, reached their maximum growth phase after 10 days of storage suggesting that they would have lost their fermentation power afterwards. The fermentation test performed using stabilized starter sampled at different growth phases, revealed that the starter of 25 days of storage possesses better fermentation capacity. In these conditions, the fermentation duration was reduced to 16 instead of 24 h.

Key words: Sorghum, opaque beer, *tchoukoutou*, starter, storage duration.

INTRODUCTION

Sorghum is an important nutrients source for millions of people in the semi-arid regions where it is a main staple (Lyumugabe et al., 2012). Cumulative to its adaptability to harsh environmental conditions, being tolerant to drought and low-input conditions, sorghum contains diverse phytochemicals with various functional properties (Shumba, 1994; Nkongoloand and Nsapato, 2003). Sorghum ranks fifth among cereals worldwide (Grubben and Partohardjono, 1996) and is directly used or transformed into various food commodities including

*Corresponding author. E-mail polykap@yahoo.fr. Tel: (+229) 97870734.

Author(s) agree that this article remains permanently open access under the terms of the Creative Commons Attribution License 4.0 International License

traditional alcoholic beverages (Dicko et al., 2006). Commonly called traditional opaque beers, these sorghum beverages play a significant role in African people cultures (Aka et al., 2008). Tchoukoutou, a traditional opaque sorghum beer of Benin, is produced, sold and consumed as street food all over the country. Like many other opaque sorghum beers in Africa, tchoukoutou has a sour taste, relatively high dry matter content (5 to 13%) and low alcohol content (2 to 3%) which make it suitable beverage for adults and teenagers (Agu and Palmer, 1998; Briggs et al., 2004). Considering the large quantity that is daily consumed, it is regarded as a significant source of dietary nutrients. Moreover, tchoukoutou significantly contributes to the diets of millions of people and generates income for the women sellers who produce it at household level, using the traditional technology (Kayode et al., 2007). Basically, the manufacturing process consists in malting, sun drying, milling, souring, boiling, mashing and alcoholic fermentation (Odunfa, 1985; Haggblade and Holzapfel, 1989). The fermentation constitutes a major step in the process. Prior to the alcoholic fermentation, the wort is inoculated with a traditional starter called *kpete-kpete* which is harvested from the previous fermenting beer. But the preservation of such a starter is a tedious and chancy business, since it may lose its fermenting properties within three days. In order to ensure quality safety and product stability, the stabilization of *kpete-kpete* in the form of a lasting starter is necessary. The procedure to stabilize *kpete-kpete* in the form of granule starter was previously described (Kayode et al., 2012). However, the storage behaviour and the functionality of such stabilized starter is not yet investigated. The objectives of the present study are two-fold. Firstly, to assess the effects of storage conditions on the physicochemical and microbiological characteristics of the stabilized starter. Secondly, to determine the maturation period of the improved starter that warrant its efficient functionality.

MATERIALS AND METHODS

Production of stabilized granule starter

Traditional starter, locally known as *kpete-kpete*, was collected from a “tchoukoutou” producer, in one commercial processing site, Abomey-Calavi town. The samples were collected in sterile bottles, packed in ice cold box and transported to the laboratory. Four kilograms of cleaned sorghum grains were dehulled using a mini-PRL dehuller (Thiés, Sénégal) and then ground. The flour obtained was mixed with distilled water (42% w/w), inoculated with 10% (w/w) of *kpete-kpete*, after being warmed up at 70°C for 15 min. The inoculated flour was allowed to ferment in a plastic bucket with lid for 24 h before being granulated. The wet granules were oven dried for 24 h at 43°C and then conditioned.

Experimental design

In order to compare the ability of both traditional and stabilized starters to preserve their functional properties during the storage, subsamples (50 g) of each of them were prepared and stored at room temperature for a period of 40 days (traditional starter) and 60 days (improved starter). Samples of the traditional starter could not be stored beyond 40 days because after this period they were totally spoiled. Samples were withdrawn every 10 days’ time and analyzed in triplicate for physicochemical and microbiological characteristics. This stage helped to identify different growth phases (latency, exponential and stationary phases) of the microorganisms. The study of the functional properties of the stabilized starters focused on their ability to ferment the sorghum wort. For this purposes, samples (50 g) of granules starters of 5 days, 25 days and 50 days of maturation corresponding to latency phase, exponential phase and stationary phase were respectively used to inoculate 1 L of sterile sorghum wort (autoclaved at 121°C for 1 h). The inoculated wort was allowed to ferment for 24 h at room temperature and samples were withdrawn at 0, 8, 16 and 24 h in triplicate for microbiological analysis.

Physico-chemical analysis

Dry matter was determined according to the AACC method (AACC, 1984). The pH was determined using a digital pH meter (HI 8418; Hanna instruments, Limena, Italy) calibrated with buffers at pH 4.0 and 7.0 (WTW, Weilheim, Germany). The titratable acidity, expressed as lactic acid, was performed by using the method described by Nout et al. (1989). The refractive index was measured using a refractometer (Sopelem 9596, France).

Microbiological analysis

Duplicate samples of starter were diluted in 90 ml sterile peptone physiological saline solution (5 g peptone, 8.5 g NaCl and 1000 ml distilled water, pH = 7.0) and homogenized with a Stomacher lab-blender (type 400, London, UK). Decimal dilutions were plated. Total counts of aerobic mesophilic (TC), lactic acid bacteria (LAB) and yeasts were enumerated as described by Hounhouigan et al. (1993). Total mesophilic aerobic bacteria counts were determined on plate count agar (PCA, oxoid, CM 325, Hampshire, England) after incubation at 30°C for 72 h. Lactic acid bacteria (LAB) were determined on Man Rogosa Sharpe Agar (MRSA, CM 361, Oxoid, Hampshire, England) with incubation in anaerobic at 30°C for 72 h. Yeasts were determined on oxytetracyclin glucose yeast extract agar (OGYA, Oxoid CM 0545, Basingstoke, Hampshire, England) containing oxytetracyclin, after incubation at 25°C for 72 h.

Data analysis

Data were analyzed with the SPSS 16.0 software (SPSS Inc., Chicago, IL, USA). Comparison of the mean values was performed applying the one-way analysis of variance (ANOVA) by using a mixed model with two factors (repetitions and type of starter).

RESULTS AND DISCUSSION

Effects of storage duration on the physicochemical characteristics of the starters

The results of ANOVA showed that the type of starter, the storage duration as well as their interactions significantly affected ($p< 0.01$) the pH, the titratable acidity and the dry matter contents of the starters (Table 1). Figure 1 showed the compared effects of storage duration on

Table 1. Results of analysis of variance on repeated measures for the traditional starter and the improved starter physicochemical and microbiological characteristics.

Sources of variation	DF	Dry matter (%)	pH	Titratable acidity (g/kg d.w)	Yeasts ($log_{10CFU\ ml}^{-1}$)	LAB ($log_{10CFU\ ml}^{-1}$)
Types of starter	1	0.002**	0.001**	0.003**	0.112	0.003**
Storage duration	4	0.015*	0.000***	0.000***	0.000***	0.000***
TS*SD	4	0.002*	0.000***	0.000***	0.000***	0.000***

DF= Degree of freedom *: significant : **: highly significatif ; ***: very highly significant; LAB: lactic acid bateria; TS: type of starter; SD: storage duration.

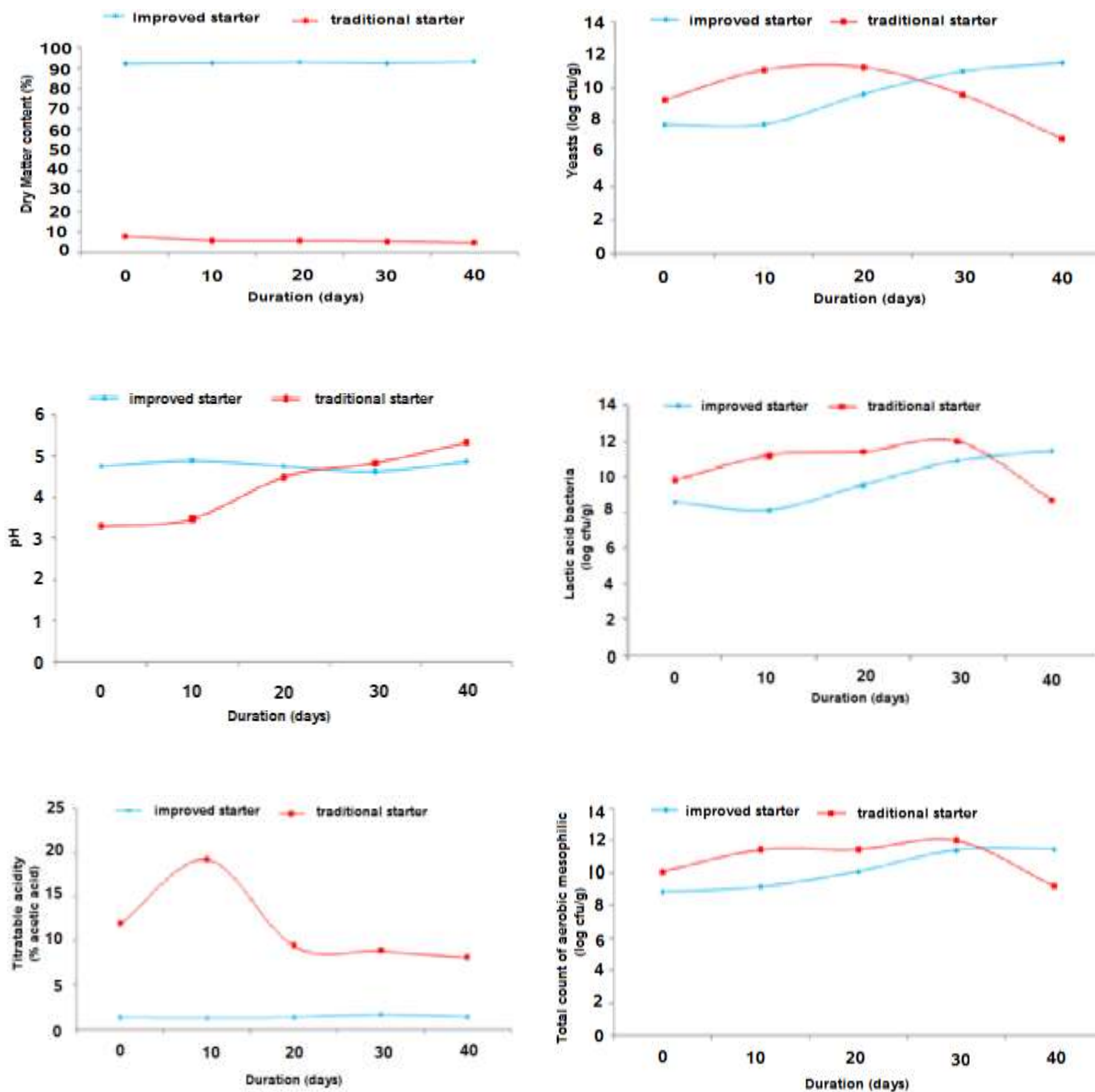


Figure 1. Changes in physicochemical and microbial properties of *kpete-kpete* (traditional) and granule starter (improved) during storage.

Table 2. Trend of Physicochemical characteristics in the improved starter over 60 days of storage

	0 day	10 days	20 days	30 days	40 days	50 days	60 days
Dry matter content (%)	92.07± 0.74^{a}	92.43±0.15^{a}	92.86±0.22^{a}	92.35±0.07^{a}	93.11±0.63^{a}	92.5±0.51^{a}	92.24±0.24^{a}
pH	4.75±0.01^{a}	4.89±0.01^{b}	4.75±0.04^{a}	4.62±0.05^{c}	4.87±0.01^{b}	4.62±0.01^{c}	4.79±0.01^{ab}
Titratable acidity (g/kg dw)	1.23±0.02^{a}	1.18±0.01^{b}	1.24±0.00^{ac}	1.60±0.01^{d}	1.28±0.01^{be}	1.43±0.01^{f}	1.32±0.01^{e}

Values with the same letter are not significantly different from each other ($p < 0.05$).

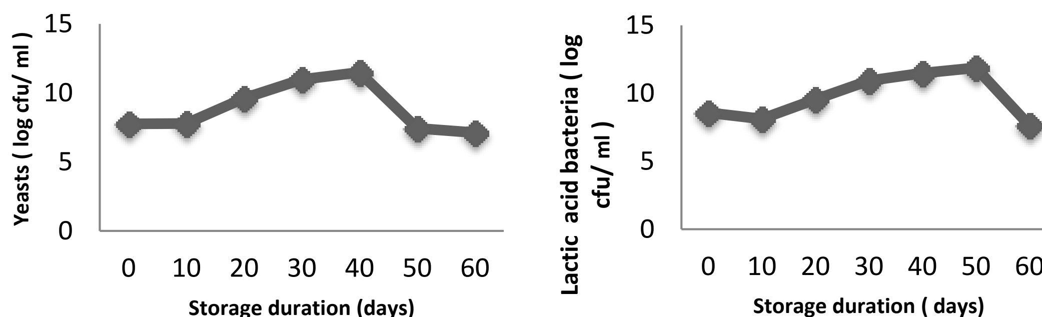


Figure 2. LAB and yeasts trend in the improved starter over 60 days of storage.

physicochemical characteristics of the traditional and improved starters. Within 10 days of storage, the dry matter content of the traditional starter significantly ($p<0.05$) decreased from 8.07 to 5.8 %. In opposite, the dry matter content of the improved starter remained stable throughout 40 days of storage. The mean value of the dry matter content of the improved starter was 92.5% which corresponds to a water content of 7.5%. Such level of humidity is desirable since it is favorable for a longer product shelf life. It was demonstrated that cereals products were preserve well with a water content < 12% (Cecil, 1992). As it shown in Figure 1, the pH value of the traditional starter increased from 3.30 to 5.30, whereas it remained quite stable in the improved starter (4.75) throughout the storage course. With respect to the titratable acidity, it also remained stable in the improved starter during the 40 days of storage, whereas it significantly increased from 12.00 to 19.30 g.kg^{-1}dw in the traditional starter within 10 days of storage. Thereafter, it gradually decreased from 19.30 to 8.20 g.kg^{-1}dw at 40 days of storage. Clearly, the results showed that the traditional starter could not be stable for more than 10 days, whereas the improved starter was stable within 40 days of storage. Moreover, the physicochemical characteristics of the improved starter were even stable up to 60 days (Table 2). Thus, the stabilized starter bears promise for a long shelf life while preserving its physicochemical properties. It had been reported that the use of stabilized starter can improve the hygienic quality and acceptability of African traditional foods (Gran et al., 2003). Such an observation was reported by Sawadogo-Lingani et al. (2008) for *dolo* in Burkina Faso, Glover et al. (2009) and Adewara et al. (2013) for *burukutu* in Nigeria, Orji et al. (2003) for *pito* in Nigeria and N'guessan et al. (2010) for *chakpalo* in Côte d'Ivoire. Thus, it could be expected that the use of the stabilized starter can contribute to reduced variations in the organoleptic and microbiological quality of African sorghum beers (Kirmaryo et al., 2002).

Effects of storage duration on the microbiological characteristics of the starters

The major groups of microorganisms involved in the fermentation of opaque sorghum beer are LAB and yeasts (Djè et al., 2009; Coulibaly et al., 2014). After the drying treatments applied, the functional microflora of the stabilized starter granule remained viable. In the improved starter stored for 60 days, yeast, LAB and total counts of aerobic mesophilic were modified following three phases: a latency phase (between 0 and 10 days), an exponential growth phase (between 10 and 40 days) and the decline phase (between 40 and 60 days) (Figure 2). The level of total counts of aerobic mesophilic, LAB, and yeasts were 8.87, 8.57 and 7.76 log cfu g^{-1}, respectively. In the traditional starter, *kpete-kpete*, as much as 10.10, 9.80 and 9.29 log cfu ml^{-1} was detected for the total counts of aerobic mesophilic, LAB and yeasts, respectively. The results of ANOVA showed that the type of starter, the storage duration as well as their interactions significantly affected ($p<0.01$) the total microbial count, the LAB and the yeasts counts of the starters (Table 1). Changes in LAB and yeasts composition of the starters during the storage process

Table 3. Results of analysis of variance showing the effects of storage duration and time of wort fermentation on yeasts and LAB contents of sorghum beer.

Source	DF	Yeasts ($log_{CFU\ ml^{-1}}$)		LAB ($log_{CFU\ ml^{-1}}$)	
		F-value	Prob. > F	F-value	Prob. > F
Storage duration (days)	2	20099.9	<0.001	5229.75	<0.001
Time of fermentation (hours)	2	157321	<0.001	294529	<0.001
Duration x time of fermentation	4	2718.20	<0.001	527.90	<0.001

DF = Degree of freedom, F-value = value Fisher's statistic, Prob.= probability of the test.

are shown in Figure 1. In the improved starter, yeasts count is stable for the first 10 days which corresponds to the latency phase. Afterwards, the yeasts level increased gradually from 7.78 log cfu g^{-1} at the 10th day of storage to 11.48 log cfu g^{-1} at the 40th day of storage. On the other hand, in the traditional starter, yeasts count increased from 9.29 log cfu ml^{-1} and reached its highest value of 11.48 log cfu ml^{-1} at the 15th day of storage. Thereafter, it started decreasing to reach a level of 6.96 log cfu ml^{-1} at the 40th day of storage. Growth trend for the LAB and total counts of aerobic mesophilic bacteria was similar to that of yeasts. Overall, the improved starter is more stable than the traditional starter in terms of physical, chemical and microbiological characteristics. The traditional starter remained stable only for 15 days while the improved starter preserved its microbiological characteristics over 60 days. Since the fermentation is the cumulative effect of the growth of yeast on wort, ultimately resulting in production of alcohol in the growth medium (Lodolo et al., 2008), the improved starter can be expected to trigger an effective fermentation process.

Effects of storage duration on the functional properties of the improved starter

Functional properties of the improved starter were assessed through its ability to ferment the sorghum wort. Thus, the effects of storage duration on the functional properties of the improved starter were evaluated using a fermentation test. In this respect, improved starter sampled of various storage durations, that is, 5, 25 and 50 days of storage, corresponding to the three growth phases were respectively used to inoculate different samples of sterile sorghum wort. The inoculated sorghum wort was allowed to ferment for 24 h. LAB and yeasts were enumerated at 0, 8, 16 and 24 h. Results of the analysis of variance showed that storage duration of the improved starter significantly affected ($p < 0.001$) the growth pattern of LAB and of yeasts during the wort fermentation. Likewise, the fermentation duration significantly affected ($p< 0.001$) the LAB and yeasts growth in the wort. Moreover, the interactive effects of starter duration x fermentation duration significantly affected ($p < 0.001$) the functional microflora concentration in the wort (Table 3). LAB and yeast were simultaneously affected by the storage duration of the improved starter, the fermentation duration and the interactive effects of starter duration x fermentation duration. A symbiotic relation between yeast and LAB in the wort was reported (Munyaja et al., 2003). LAB created an acid environment favorable to the proliferation of yeasts which produce vitamin and increase other factors such as amino-acids for the growth of LAB (Lyumugabe et al., 2010). Holzapfel (1997) reported that African opaque beers are typically characterized by a lactic fermentation followed by an alcoholic fermentation in which initially, LAB and later yeasts play the dominant role. Due to their higher growth rate, bacteria typically dominate the early stages of fermentation. Clearly, yeasts grew faster in the wort inoculated with starter of 25 days of storage than in wort inoculated with starters of 5 and 50 days of storage. At 16 h of fermentation, the maximal value of yeasts count (9.36 log cfu ml^{-1}) was reached in worts inoculated with 25 days starter; whereas in worts inoculated with days and 50 days matured starters, it took 24 h of fermentation to reach the maximum of this value (6.67 and 7.74 log cfu ml^{-1}, respectively) (Figure 3). Similar trends were observed for LAB count. At 16 h of fermentation, the count of LAB in the wort inoculated with the starter of 25 days storage (8.0 log cfu ml^{-1}) was significantly ($p<0.001$) higher than values obtained in worts inoculated with starters of 5 days (5.5 log cfu ml^{-1}) and 50 days of storage (5.5 log cfu ml^{-1}). The use of the starter of 25 days permitted to reduce the fermentation duration to 16 h instead of 24 h. Similar findings were obtained by some authors. Sawadogo-Lingani et al. (2008) tested three kinds of improved starter cultures for dolo (sorghum beer in Burkina Faso) and found a reduction of the fermentation duration. The same with N'guessan et al. (2010) for the tchakpalo (sorghum beer in Côte d'Ivoire) and Orji et al. (2003) for pito (sorghum beer in Nigeria).

Conclusion

The heat stabilized *kpete-kpete*, in the form of granules, had preserved physicochemical and microbiological characteristics and functional properties better than the

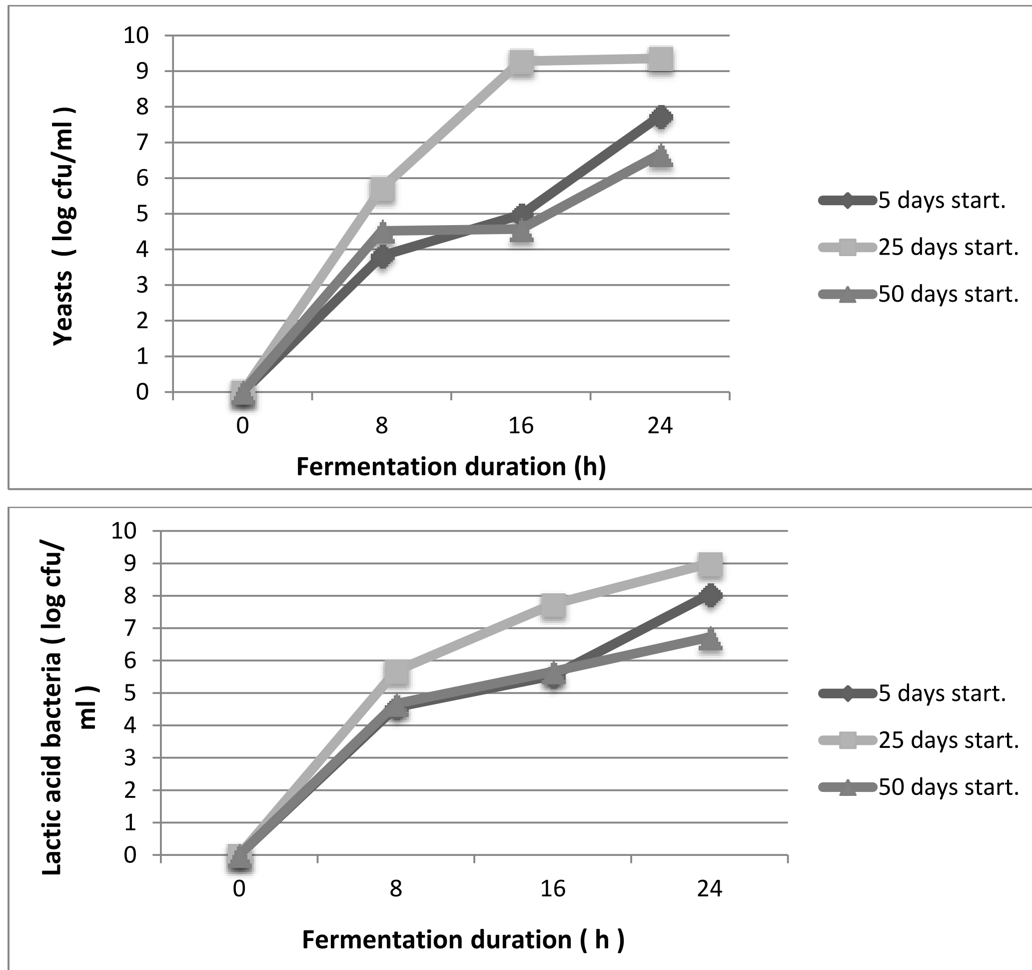


Figure 2. Fermentation efficacy of starter granules sampled at various storage times as established after inoculation in sterile sorghum wort.

traditional starter. The improved starter reached its maturation at 25 days of storage where its ability to ferment the sorghum wort was significantly better than that of starters stored for 5 or 50 days. Therefore, the 25 days stored starter could be used to produce a sorghum beer at shorter fermentation time with adequate physicochemical and microbiological properties.

Conflict of interests

The authors have not declared any conflict of interests.

REFERENCES

AACC (1984). Approved methods of the American Association of cereal chemistry. American Association of Cereal Chemistry, St. Paul, MN.

Adewara AO, Ogunbanwo ST (2013). Effects of processing variables on the production of “Burukutu”, a Nigerian fermented beverage. Nat. Sci. 11:16-28.

Agu RC, Palmer GH (1998). A reassessment of sorghum for lager-beer brewing. Bioresour. Technol. 66:253- 261.

Aka S, Djeni NT, N’guessan KF, Yao KC, Dje KM (2008). Variabilité des propriétés physico-chimiques et dénombrement de la flore fermentaire du tchapalo, une bière traditionnelle de sorgho en Côte d’Ivoire. Afrique SCI. 04:274-286.

Briggs DE, Boulton CA, Brookers PA, Stevens R (2004). Native African beers. In Brewing: Science and practice.Woodhead publishing Ltd, Cambridge, UK. Pp.589-605.

Cecil SE (1992). Semiwet milling of red sorghum. In Utilization of sorghum and millet. M.I. Gomez, L.R., House, L.W. Rooney and D.A.V. Dendey, eds. ICRISAT. pp. 25-36.

Coulibaly WH, N’guessan KF, Coulibaly I, Djè KM, Thonart P (2014). Les levures et les bactéries lactiques impliquées dans les bières traditionnelles à base du sorgho produites en Afrique Subsaharienne. Biotechnol. Agron. Soc. Environ. 18(2):209-219.

Djè MK, N’guessan FK, N’dede DT, Dadie TA (2008). Biochemical changes during alcoholic fermentation in the production of tchapalo, a traditional sorghum beer. Int. J. Food Eng. 4 (7).

Djè KM, N’guessan FK, N’dede DT, Dadie TA (2009). Predominant lactic acid bacteria involved in the spontaneous fermentation step of tchapalo process, a traditional sorghum beer of Côte d’Ivoire. Res. J. Biol. Sci. 4(7):789-795.

Dicko MH, Gruppen H, Traoré1 AS, Voragen AGJ, Van Berkel WJH (2006). Sorghum grain as human food in Africa: relevance of content of starch and amylase activities. Afr. J. Biotechnol. 5:384-395.

Glover RLK, Sawadogo-Lingani H, Diawara B, Jespersen L, Jakobsen M (2009). Utilization of *Lactobacillus fermentum* and *Saccharomyces*

*cerevisiae*as starter cultures in the production of “dolo”. J. Appl. Biosci. 22:1312-1319.

Gran HM, Gadaga HT, Narbhus JA (2003). Utilization of various starter cultures in the product of Amasi, a Zimbabwean naturally fermented raw milk product. Int. J. Food Microbiol. 88:19-28.

Grubben GJH, Partohardjono S (1996). Plant Resources of South-east Asia. Backhuys Publishers, Leiden. Pp.122-128.

Haggblade S, Holzapfel H (1989). Industrialization of indigenous fermented foods. Steinkraus K.H. Ed. Marcel Dekker, New York. p.9.

Holzapfel W (1997). Use of starter cultures in fermentation on a household scale. Food Control. 8:241-258.

Hounhouigan DJ, Nout MJR, Nago CM, Houben JH, Rombouts FM (1993). Microbiological changes in mawe during natural fermentation. World J. Microbiol. Biotechnol.10: 410 -413.

Kayodé APP, Hounhouigana JD, Nout MJR (2007). Impact of brewing process operations on phyta, phenolic compounds and in vitro solubility of iron and zinc in opaque sorghum beer. LWT. 40:834-841.

Kayode APP, Deh DC, Baba-Moussa L, Kotchoni SO, Hounhouigan JD (2012). Stabilisation and preservation of probiotic properties of the traditional starter of African opaque sorghum beers. Afr. J. Biotechnol. 11(30):7725 -7730.

Kirmaryo VM, Massawe GA, Olasupo NA, Hololzpfel WH (2002). The use of a starter culture in the fermentation of cassava for the production of ‘’ Kivunde’’, a traditional Tanzanian food product. Int. J. Food Microbiol. 56:179-190.

Lodolo EJ, Kock JLF, Axcell BC, Brooks M (2008).The yeast *Saccharomyces cerevisiae*-the main character in beer brewing. FEMS Yeast Res. 8:1018-1036.

Lyumugabe F, Kamaliza G, Bajyana E, Thonart PH (2010). Microbiological and physic-chemical characteristics of Rwandese traditional beer "Ikigage"". Afr. J. Biotechnol. 9:4241-4246.

Lyumugabe F, Gros J, Nzungize J, Bajyana E, Thonart PH (2012). Characteristics of African traditional beers brewed with sorghum malt: a review. Biotechnol. Agron. Soc. Environ. 16(4):509-530.

N’guessan FK, N’Dri DY, Camara F, Dje MK (2010).*Saccharomyces cerevisiae* and *Candida tropicalis* as starters cultures for the alcoholic fermentation of tchapalo, a traditional sorghum beer. World J. Microbiol. Biotechnol. 26:693-699.

Nkongolo KK, Nsapato L (2003). Genetic diversity in *Sorghum bicolor* (L.) Moench accessions from different ecogeographical regions in Malawi assessed with RAPDs. Genet. Resour. Crop 50:149-156.

Odunfa SA (1985). African fermented foods. In: Wood, BJB. (ed.), Microbiology of Fermented Foods. Elsevier Applied Science, London, UK. pp. 167-195.

Orji MU, Mbata TI, Aniche GN, Ahonkhai I (2003).The use of starter cultures to produce “Pito”, a Nigerian fermented alcoholic beverage. World J. Microbiol. Biotechnol. 19:733-736.

Pale S, Taonda SJB, Bougouma B, Mason SC (2010). Sorghum malt and traditional beer (Dolo) quality assessment in Burkina Faso. Ecol. Food Nutr. 49:129-141.

Nout MJR, Rombouts FM, Havelaar A (1989). Effect of accelerated natural lactic fermentation of infant food ingredients on some pathogenic micro-organisms. Int. J. Food Microbiol. 8:351-361.

Sawadogo-Lingani H, Diawara B, Traoré AS, Jakobsen M (2008). Utilisation de souches sélectionnées de *Lactobacillus fermentum* et un isolat de levure comme cultures starter dans la production du dolo, une boisson fermentée à base de sorgho. Sci. Technol. 2:61-84.

Shumba D, Leuschner L, Manthe CS (1994). Promotion of a small grains seed production by a nongovernmental organization in Zimbabwe. In Drought-Tolerant Crops for Southern Africa: Proceedings of the SADC/ICRISAT Regional Sorghum and Millet Quality Workshop. International Crops Research Institute for the Semi-Arid Tropics: Patancheru, India, pp. 227-234.

academicJournals

Vol. 10(43), pp. 1829-1836, 21 November, 2016
DOI: 10.5897/AJMR2016.8343
Article Number: 2E479A461665
ISSN 1996-0808
Copyright © 2016
Author(s) retain the copyright of this article
http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Full Length Research Paper

Selection of anti-wetting agents and photoprotectants compatible with a soybean weed control bioagent

Machado, A. C. R.*, Monteiro, A. C. and Mochi, D. A.

Post-Graduate Program in Agricultural and Livestock Microbiology, Faculty of Agricultural and Veterinary Sciences, Univ Estadual Paulista, Campus of Jaboticabal, 14884-900 Jaboticabal, SP, Brazil.

Received 18 October 2016, Accepted 9 November, 2016.

Adjuvants incorporation at formulations can positively influence the performance of bioagent control, contributing to preservation of the inoculum until being used. Adjuvants should not be toxic to pathogen and tests that evaluate the sensitivity of biologic products are essential. The aim of this study was to analyze the compatibility of the anti-wetting agents microcrystalline cellulose, anhydrous sodium carbonate, magnesium oxide, talc, calcium carbonate, silicon dioxide, and photoprotectants NeoHeliopan® AV, NeoHeliopan® E1000, Eusolex® 6007, NeoHeliopan® Hydro, Tinosorb® M, Eusolex® 232 and a Complex filters UVA/UVB with the fungus *Bipolaris euphorbiae*, in order to select products to formulate a fungus-based bioherbicide. All products were used in concentrations of 0.01, 0.05, 0.1, 0.5 and 1%. After evaluating vegetative growth, sporulation, and germination, the products were toxicologically classified by calculating the biological index. The anti-wetting agents calcium carbonate, talc, microcrystalline cellulose, and silicium dioxide did not interfere in the development of the fungus, and were deemed compatible. The photoprotectant Tinosorb® M was classified as compatible with the fungus in all concentrations used, a similar outcome to Eusolex® 6007, except at 1.0% concentration. Most of the other photoprotectants were compatible in concentrations ranging between 0.01 and 0.1%, except for Complex filters UVA/UVB at 0.05% and NeoHeliopan® E1000 at 0.1%. NeoHeliopan® Hydro was rated moderately toxic to the fungus in all concentrations used. Conidial germination was less affected than growth and sporulation.

Key words: *Bipolaris euphorbiae*, biological control, bioproduct, formulation, adjuvants.

INTRODUCTION

Bipolaris euphorbiae is a specific pathogen of *Euphorbia heterophylla* L. (milkweed), one of the most significant weeds affecting soybeans. In order to be used as a biological control agent, it is necessary to develop a fungus-based bioproduct.

Development of bio-herbicides requires finding appropriate technologies for mass production, formulation, and preservation of the inoculum until the use phase (Tessmann, 2011). One of the aspects that limits the advances in this type of control is obtaining appropriate

*Corresponding author. E-mail: biokarol@hotmail.com. Tel: +98 513 8012589.

Author(s) agree that this article remains permanently open access under the terms of the Creative Commons Attribution License 4.0 International License

Table 1. Chemical adjuvants assessed in this study with respect to compatibility with *Bipolaris euphorbiae*.

Function	Trade name	Chemical name or composition
Anti-wetting agent	Microcrystalline cellulose	-
	-	Anhydrous sodium carbonate
	-	Magnesium oxide
	Talc	Hydrated magnesium silicate
	-	Calcium carbonate
	-	Silicium dioxide (Pyrogenic silica)
Photoprotectant	NeoHeliopan® AV	Octyl methoxycinnamate
	NeoHeliopan® E1000	Isoamyl-methoxycinnamate
	Eusolex® 6007	Ethylhexyl dimethyl PABA
	NeoHeliopan® Hydro (Symrise)	Phenylbenzimidazole sulfonic acid
	Tinosorb® M	Methylene-bis-benzotriazolyl tetramethylbutylphenol
	Eusolex® 232 (Merck)	Phenylbenzimidazole sulfonic acid
	Complex filters UVA/UVB	Benzophenone 4 + phenylbenzimidazole sulfonic acid

formulations. Formulation is the way in which the active ingredient is presented in an effective physical form. It begins with the production of the pathogen, and continues through the addition of products (adjuvants) that aim to stabilize the biological agent during storage, facilitate product handling and application, protect the bioagent from environmental factors, and increase pathogen activity, boosting its reproduction, contact and interaction with the target host (Morentini and Melo, 2007; Almeida et al., 2008).

Mass production of *B. euphorbiae* and the pre-stages in obtaining a bio-herbicide have already been investigated and established (Penariol et al., 2008a; Machado et al., 2013; Moraes et al., 2014). However, it is important to find adjuvants compatible to the pathogen which promotes improvements in one or more characteristics essential to establishing the antagonistic relationship (Fravel et al., 1998) that favor the development of a formulation. Among adjuvants that are usable in formulating myco-herbicides, anti-wetting agents that keep the level of available water low, that prevent fungus conidia from germination when stored, stand out. Therefore, in order for a bioproducts to be commercially competitive, it is crucial to extend their useful life, to increase the period that the pathogen propagules can be stored, while remaining viable and infectious (Elzein et al., 2008).

Solar radiation is one of the most significant environmental problems affecting the efficiency of fungi as biological control agents (Braga et al., 2006), since sensitivity to radiation limits the use of such biological control agents in field conditions (Francisco et al., 2008). When conidia are exposed to ultraviolet radiation (UV), the cytoplasmic membrane and cellular organelles are subject to degradation, along with several other direct effects on the DNA, resulting in delayed germination or complete inactivation (Rangel et al., 2006; Chelico and Khachatourians, 2008). Photoprotectants are substances that are able to absorb and/or disperse UV rays, according to their structure, protecting against the adverse effects of this radiation. Therefore, it is important to find photoprotectants that are compatible with *B. euphorbiae* so they can be added to the formulation.

Adding adjuvants to formulations can influence conidia performance positively. However, the adjuvants may be toxic to the biological control agent, so it is essential to evaluate toxicity to the specific microorganism (Wyss et al., 2004).

The aim of this study was to analyze the compatibility of anti-wetting agents and photoprotectants with fungus *B. euphorbiae,* aiming to select products to formulate a fungus-based bio-herbicide.

MATERIALS AND METHODS

For use in the tests, the FCAV#569 isolate of *B. euphorbiae* was grown in Petri dishes containing the Pontecorvo minimal medium, modified by supplementation with peptone (2 g L^{-1}) and with the substitution of glucose by starch (10 g L^{-1}) (Penariol et al., 2008b). The fungus was kept at 25 ± 0.5°C for 10 days, with a 12-h photoperiod.

The adjuvants assessed and their functions are shown in Table 1. All products were used in arbitrarily defined concentrations of 0.01, 0.05, 0.1, 0.5 and 1%. Vials containing the liquid Pontecorvo minimal medium, received predetermined quantities of the adjuvants, and next, the media were transferred into Petri dishes. Since they are liposoluble, the photoprotectants NeoHeliopan® AV, NeoHeliopan® E1000 and Eusolex® 6007 were mixed in sterile solution of arabic gum (0.7% w v^{-1}) for emulsification before being added to the culture medium. To increase solubilization and stabilize pH, the photoprotectants NeoHeliopan® Hydro and Eusolex® 232 were added along with Triethanolamine (1:1 v v^{-1}), which was pre-tested for compatibility with the fungus. The control treatment was composed of the minimal medium without the addition of any product. After the culture medium solidified, the inoculation was conducted, by transferring to the center of the Petri

dish a 5 mm diameter disc of fungus culture obtained from colonies with 10 days of growth. Then, *B. euphorbiae* colonies were maintained in the incubation conditions described earlier.

The vegetative growth, sporulation, and conidia germination were assessed. Vegetative growth was analyzed by measuring (in mm) two perpendicular diameters on the 10th day of incubation. After this period, the conidia formed on the surface of the colony were removed by scraping and transferred to a test tube containing 9 ml of Tween 80® solution (0.1% v v^{-1}). From this suspension, conidia number was determined by counting in a Neubauer chamber. Germination was assessed by micro-cultivation on slides and direct microscopic examination, according to the methodology described by Francisco et al. (2006). Three areas were marked microscope slides, and the surface was covered with 4 ml of minimal medium containing the products in the respective concentrations. In the region of the culture medium, a drop of a fungal suspension (1 × 10^6 con. ml^{-1}) was inoculated, and incubated at 25 ± 0.5°C for 7 h. One hundred and fifty conidia were observed, germinated and non-germinated, in each area, thus establishing a percentage ratio of viable conidia.

Tests were conducted using a completely randomized design (CRD) composed of 4 repetitions. Data were subjected to variance analysis using the F test, and the means were compared using the Tukey test at 5% probability. The AgroEstat program was used for the statistical tests

To determine the toxicity of the adjuvants for the fungus, the biological index (BI) model was used, which is described by Rossi-Zalaf et al. (2008) and calculated by the formula:

$$BI = \frac{47[VG] + 43[SP] + 10[GER]}{100}$$

where VG = percentage of vegetative growth in the colony after 10 days of incubation as compared to the control, SP = percentage of sporulation after 10 days of incubation compared to the control, GER = percentage of germination of the conidia after 7 h of incubation, in relation to the control.

Using the BI values, adjuvants toxicity was classified using the scale described by Rossi-Zalaf et al. (2008), where BI from 0 to 41 = toxic; BI from 42 to 66 = moderately toxic; and BI > 66 = compatible.

RESULTS AND DISCUSSION

Different concentrations of the anti-wetting agents calcium carbonate, talc, microcrystalline cellulose, and silicon dioxide did not affect the development of *B. euphorbiae,* especially with regard to vegetative growth and germination, being considered compatible with the fungus (Tables 2 and 3). Therefore, these products can be used in formulating fungus-based bio-herbicides at any of the tested concentrations.

In the treatments containing 0.5 and 1% of anhydrous sodium carbonate and magnesium oxide, all of the evaluated parameters were affected, but the germination obtained in the presence of magnesium oxide (>93%) can be considered satisfactory (Tables 2 and 3, respectively), suggesting that initially the fungus used endogenous sources of macronutrients that allowed the germination of conidia. Then they used exogenous sources contained in the culture medium with the anti-wetting and causing the deleterious effect.

In these concentrations, both products were considered toxic to the fungus. Anhydrous sodium carbonate was classified as compatible with the fungus only in concentrations of 0.01 and 0.05% (Table 2), and the magnesium oxide was deemed compatible in concentrations of 0.01 to 0.1%, as it had little or no significant effect on the performance of the bioagent (Table 3).

The development of formulations based on biological agents is very similar to processes in the pharmaceutical, cosmetics, and food industries, in terms of searching for ingredients that are safe, inexpensive, and non-toxic (Gaugler, 1997). The products tested in this study are commonly used in the food industry and are classified under Brazilian law as safe for human consumption.

Results of studies addressing fungi pathogenic to weeds associated with anti-wetting agents for use in formulating bio-herbicides have not been found in the literature, which makes it impossible to compare the results obtained in this study with those of other authors. Therefore, these results are important because they represent the first data obtained for these topics.

Among the parameters used to evaluate the compatibility of photoprotectants with *B. euphorbiae*, sporulation of the fungus was more affected than the control (Tables 4 and 5). Growth and germination were less affected by the action of most of the products tested, although there were significant differences compared to the control in some treatments.

NeoHeliopan® AV was classified as compatible with the fungus when used at 0.01, 0.05 and 0.1%, but completely inhibited growth, sporulation, and germination at concentrations of 0.5 and 1.0%, and was classified as toxic (Table 4). The chemical derivative contained in this commercial product is one of the most commonly used to protect against electromagnetic spectrum UVB radiation, and Brazilian law permits its use at concentrations varying from 2 to 7.5% for pharmaceutical formulations. The concentrations used in the present study, were considerably lower than these levels, but the product was only compatible with the fungus up to 0.1% concentration (Table 4).

The photoprotectant NeoHeliopan® E1000 affected the performance of the fungus in concentrations of 0.1%to 1.0%, and was classified as moderately toxic, but proved to be compatible at concentrations of 0.01 and 0.05% (Table 4). Eusolex® 6007 showed no deleterious effect on growth and germination, affecting only sporulation. Consequently, it was classified as compatible with the fungus at all concentrations, except 1.0% (Table 4). NeoHeliopan® Hydro did not affect germination of *B. euphorbiae*, had little effect on growth, and most impacted sporulation. Consequently, it was classified as moderately toxic to the fungus at all of the assessed concentrations (Table 5). In the presence of Tinosorb® M, growth, sporulation, and germination of *B. euphorbiae* were unaffected, with no difference in relation to the control. Based on these data, the calculation of the

Table 2. Toxicity of the anti-wetting agents calcium carbonate, talc, and anhydrous sodium carbonate to *B. euphorbiae* grown in culture medium containing different concentrations of products.

Anti-wetting agents and concentrations	Growth (mm)	Sporulation (n° of con. × 10^6)	Germination (%)	BI	Toxicological classification
Calcium carbonate					
Control	90.0	4.8^A	95.6^A	-	-
0.01%	90.0	3.6^A	93.5^{AB}	90	C
0.05%	90.0	8.0^A	94.9^{AB}	129	C
0.1%	90.0	4.8^A	94.4^{AB}	100	C
0.5%	90.0	3.6^A	95.0^A	79	C
1.0%	90.0	6.3^A	92.2^B	113	C
F test	-	2.44^{ns}	4.74*	-	-
C.V. (%)	-	9.26	1.56	-	-
Talc					
Control	90.0	10.5^A	99.9^A	-	-
0.01%	90.0	7.0^{AB}	99.6^A	86	C
0.05%	90.0	8.0^{AB}	99.6^A	90	C
0.1%	90.0	2.5^C	99.9^A	67	C
0.5%	90.0	4.5^{BC}	99.8^A	75	C
1.0%	90.0	4.0^{BC}	99.9^A	77	C
F test	-	5.67**	0.47^{ns}	-	-
C.V. (%)	-	10.61	2.45	-	-
Anhydrous sodium carbonate					
Control	90.0^A	9.0^A	99.8^A	-	-
0.01%	90.0^A	4.3^B	99.0^A	67	C
0.05%	90.0^A	4.5^B	98.6^A	78	C
0.1%	90.0^A	1.1^C	99.3^A	62	MT
0.5%	20.0^B	0.2^C	62.4^B	18	T
1.0%	0.0^C	0.0^C	7.7^C	1	T
F test	45844.64**	20.52**	791.43**	-	-
C.V. (%)	0.62	9.16	2.60	-	-

Original values and statistical analysis of sporulation and germination performed with data transformed into log x and arc sin (x/100), respectively. Means followed in the column by at least one common letter do not differ by the Tukey test ($p \geq 0.05$). ^{ns}Not significant; ** and *Significant at 1 and 5% probability, respectively. BI: Biological index. CV: coefficient of variation. C: compatible; MT: moderately toxic; T: toxic.

biological index ranged from 81 to 95, classifying this product as compatible with the fungus in all concentrations tested (Table 5).

This photoprotectant is considered to be a cutting-edge product, and is widely used around the world. It is an organic solar filter made of microfine particles whose protective action against UV radiation consists of absorption, reflection and dispersion of solar radiation (Lim et al., 2005). Furthermore, it is photostable, an important feature in developing formulations containing solar filters, since they can interact with other compounds in the formulation that may be degraded as a result of UV exposure (Wissing and Muller, 2001). Eusolex® 232 and the Complex filters UVA/UVB had little or no effect on growth and germination, and were classified as compatible with the fungus in concentrations of 0.01 to 0.1%, except for the Complex filters UVA/UVB at 0.05%. In this concentration, the product was classified as moderately toxic, with a BI of 66, a value corresponding to the maximum limit for this classification, according to Rossi-Zalaf et al. (2008), and near the lower limit for classification as compatible. In other concentrations, both products were considered moderately toxic to the fungus, and sporulation was the most affected parameter (Table 5). Studies involving the compatibility of *B. euphorbiae* with photoprotectants were not found in the literature. Various concentrations of the photoprotectants Oxybenzone®, NeoHeliopan® AV and NeoHeliopan® E1000 had no deleterious effects on the germination of *Beauveria bassiana* conidia, and were considered

Table 3. Toxicity of the anti-wetting agents magnesium oxide, microcrystalline cellulose and silicon dioxide to *B. euphorbiae* grown in culture medium containing different concentrations of products.

Anti-wetting agents and concentrations	Growth (mm)	Sporulation (nº of con. × 10^6)	Germination (%)	BI	Toxicological classification
Magnesium oxide					
Control	90.0^A	5.8^A	99.9^A	-	-
0.01%	90.0^A	6.0^A	99.5^A	102	C
0.05%	90.0^A	7.3^A	99.7^A	111	C
0.1%	90.0^A	5.5^A	93.6^B	97	C
0.5%	23.3^B	0.2^B	93.2^B	23	T
1.0%	6.8^C	0.0^B	93.7^B	13	T
F test	56.64**	15.33**	74.18**	-	-
C.V. (%)	11.87	9.62	1.63	-	-
Microcrystalline cellulose					
Control	90.0	1.7^A	99.6^A	-	-
0.01%	90.0	1.4^{AB}	99.5^A	93	C
0.05%	90.0	1.6^A	99.7^A	103	C
0.1%	90.0	1.3^{AB}	99.6^A	90	C
0.5%	90.0	1.1^{AB}	99.5^A	84	C
1.0%	90.0	0.9^B	99.1^A	88	C
F test	-	4.12*	0.23^{ns}	-	-
C.V. (%)	-	11.46	2.66	-	-
Silicon dioxide					
Control	90.0	1.65^A	98.2^A	-	-
0.01%	90.0	1.68^A	97.2^A	100	C
0.05%	90.0	0.93^A	97.0^A	81	C
0.1%	90.0	0.90^A	97.6^A	80	C
0.5%	90.0	0.85^A	97.9^A	69	C
1.0%	90.0	0.78^A	97.4^A	77	C
F test	-	3.39*	1.12^{ns}	-	-
C.V. (%)	-	3.54	1.46	-	-

Original values and statistical analysis of sporulation and germination performed with data transformed into log x and arc sin (x/100), respectively. Means followed in the column by at least one common letter do not differ by the Tukey test (p ≥ 0.05). ^{ns}Not significant; $^{**\ and}$ *Significant at 1 and 5% probability, respectively. BI: Biological index; CV: coefficient of variation; C: compatible; MT: moderately toxic; T: toxic.

compatible with the fungus (Santos et al., 2011). However, this assessment did not consider the BI model, which includes data on vegetative growth and sporulation.

The adverse effects of solar radiation on germination of conidia have been reported by several authors (Rangel et al., 2004; Rangel et al., 2005; Braga et al., 2006). These effects reduce the activity of the fungus on the host (Rangel et al., 2006; Chelico and Khachatourians, 2008). Conidia of *B. euphorbiae* are tolerant to solar and ultraviolet radiation, and remain viable (germination > 92%) after 8 h of exposure to radiation emitted by a solar simulator or 90 min of exposure to germicidal UV radiation, without the use of any formulation (Moraes et al., 2011).

Among the protective factors to UV radiation intrinsic to conidia of fungi are the pigments located in the cell wall, which act to block the entrance of radiation (Rangel et al., 2005; Braga et al., 2006). Hyphae and conidia of fungi in the genus *Bipolaris* feature dark coloration due to the presence of melanin in the cell wall (Weikert-Oliveira et al., 2002). In microorganisms, the primary function of this pigment is to reduce the harmful effects of UV radiation on the cells. The correlation between melanin concentration and UV tolerance is being discussed. Melanin is also associated with protection against high temperatures and chemical stresses such as the presence of heavy metals and oxidizing agents (Allam and Abd El-Zaher, 2012). Although the results achieved by Moraes et al. (2011) have shown that *B. euphorbiae* is quite tolerant

Table 4. Toxicity of the photoprotectants NeoHeliopan® AV, NeoHeliopan® E1000 and Eusolex® 6007 to *Bipolaris euphorbiae* grown in culture medium containing different concentrations of products.

Photoprotectants and concentrations	Growth (mm)	Sporulation (nº of con. × 10^6)	Germination (%)	BI	Toxicological classification
NeoHeliopan®AV					
Control	90.0^{A}	1.6^{A}	88.3^{A}	-	-
0.01%	90.0^{A}	0.9^{AB}	80.0^{B}	80	C
0.05%	90.0^{A}	1.8^{A}	88.1^{A}	107	C
0.1%	60.8^{B}	1.7^{A}	84.3^{AB}	91	C
0.5%	0.0^{C}	0.0^{B}	0.0^{C}	0	T
1.0%	0.0^{C}	0.0^{B}	0.0^{C}	0	T
F test	164.14**	6.75**	1129.97**	-	-
C.V. (%)	12.51	6.06	3.02	-	-
NeoHeliopan® E1000					
Control	90.0^{A}	1.7^{A}	95.6^{A}	-	-
0.01%	90.0^{A}	1.4^{AB}	94.4^{AB}	92	C
0.05%	90.0^{A}	1.2^{ABC}	90.5^{B}	87	C
0.1%	63.3^{C}	0.6^{BC}D	81.9^{C}	56	MT
0.5%	66.8^{B}	0.3^{C}D	79.5^{C}	50	MT
1.0%	65.8^{B}	0.2D	78.1^{C}	48	MT
F test	559.03**	8.32**	39.26**	-	-
C.V. (%)	0.75	3.55	2.77	-	-
Eusolex® 6007					
Control	90.0	1.6^{A}	97.9^{A}	-	-
0.01%	90.0	1.0^{AB}	98.7^{A}	95	C
0.05%	90.0	0.7^{BC}	98.3^{A}	75	C
0.1%	90.0	0.6^{BC}	97.7^{A}	73	C
0.5%	90.0	0.4^{BC}	96.8^{A}	67	C
1.0%	90.0	0.2^{C}	96.8^{A}	62	MT
F test	-	8.50**	2.32^{ns}	-	-
C.V. (%)	-	2.93	1.99	-	-

Original values and statistical analysis of sporulation and germination performed with data transformed into log x and arc sin (x/100), respectively. Means followed in the column by at least one common letter do not differ by the Tukey test ($p \geq 0.05$). ^{ns}Not significant; **Significant at 1% probability. BI: Biological index. CV: coefficient of variation; C: compatible; MT: moderately toxic; T: toxic.

of solar radiation, it is useful to combine this phytopathogen with photoprotectants in order to increase its efficiency in field conditions, since sun exposure may surpass 8 h. A wide range of photoprotectants with potential for use in formulating bioproducts is available on the market, but studies investigating the compatibility of these photoprotectants with fungal control agents are scarce, especially weed pathogens.

Conclusions

B. euphorbiae is affected by the effect of anti-wetting agents and photoprotectants which can be used as adjuvants in formulating a fungus-based bioproduct. The development of the fungus is influenced by the concentration of the products used. Conidia germination is less affected by the products than vegetative growth and sporulation. In concentrations that are not toxic or moderately toxic to the fungus the anti-wetting agents and photoprotectants tested can be used to formulate a fungus-based bio-herbicide

Conflict of interests

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENTS

The authors thank the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) for the PhD scholarship granted to the first author.

Table 5. Toxicity of the photoprotectants NeoHeliopan® Hydro, Tinosorb® M, Eusolex® 232 and Complex filters UVA/UVB to *Bipolaris euphorbiae* grown in culture medium containing different concentrations of products.

Photoprotectants and concentrations	Growth (mm)	Sporulation (nº of con. × 10^6)	Germination (%)	BI	Toxicological classification
NeoHeliopan® Hydro					
Control	99.0^{A}	7.8^{A}	87.7^{A}	-	-
0.01%	85.5^{AB}	4.1^{BC}	86.2^{A}	64	MT
0.05%	84.6^{BC}	3.5^{BC}	81.8^{A}	62	MT
0.1%	77.3^{D}	6.1^{B}	81.4^{A}	64	MT
0.5%	79.8^{CD}	1.3^{D}	81.8^{A}	52	MT
1.0%	78.3^{D}	2.1^{CD}	80.4^{A}	55	MT
F test	164.14**	22.54**	1.21^{ns}	-	-
C.V. (%)	12.51	9.22	6.15	-	-
Tinosorb® M					
Control	90.0	3.1^{A}	98.2^{A}	-	-
0.01%	90.0	1.7^{A}	98.8^{A}	81	C
0.05%	90.0	1.9^{A}	98.4^{A}	83	C
0.1%	90.0	$2,7^{A}$	98.7^{A}	95	C
0.5%	90.0	2.3^{A}	98.8^{A}	89	C
1.0%	90.0	1.8^{A}	98.7^{A}	82	C
F test	-	0.66^{ns}	0.30^{ns}	-	-
C.V. (%)	-	8.79	2.28	-	-
Eusolex® 232					
Control	90.0^{A}	1.8^{A}	97.6^{AB}	-	_
0.01%	90.0^{A}	0.8^{BC}	98.3^{A}	75	C
0.05%	90.0^{A}	0.7^{BC}	97.4^{AB}	75	C
0.1%	90.0^{A}	1.1^{AB}	97.4^{AB}	83	C
0.5%	90.0^{A}	0.4^{C}	96.6^{B}	55	MT
1.0%	65.5^{B}	0.3^{C}	97.0^{AB}	50	MT
F test	5461.68**	12.36**	2.32^{ns}	-	-
C.V. (%)	0.65	2.53	1.99	-	-
Complex filters UVA/UVB					
Control	90.0^{A}	3.2^{A}	98.4^{A}	-	-
0.01%	90.0^{A}	0.9^{B}	98.8^{A}	76	C
0.05%	90.0^{A}	0.7^{B}	98.9^{A}	66	MT
0.1%	90.0^{A}	0.8^{B}	98.6^{A}	68	C
0.5%	90.0^{A}	0.4^{B}	98.6^{A}	62	MT
1.0%	81.0^{B}	0.5^{B}	98.7^{A}	59	MT
F test	152.78**	5.82**	0.08^{ns}	-	-
C.V. (%)	0.34	6.42	2.13	-	-

Original values and statistical analysis of sporulation and germination performed with data transformed into log x and arc sin (x/100), respectively. Means followed in the column by at least one common letter do not differ by the Tukey test ($p \geq 0.05$). ^{ns}Not significant; **Significant at 1% probability. BI: Biological index; CV: coefficient of variation; C: compatible; MT: moderately toxic; T: toxic.

REFERENCES

Allam NG, Abd El-Zaher EHF. (2012). Protective role of *Aspergillus fumigatus* melanin against ultraviolet (UV) irradiation and *Bjerkandera adusta* melanin as a candidate vaccine against systemic candidiasis. Afr. J. Biotechnol. 11:6566-6577.

Almeida JEM, Batista Filho A, Alves, SB, Leite GL, Neves P M J O (2008). Formulação de entomopatógenos na América Latina. In: S. B. Alves & R. B. Lopes (Eds), Controle Microbiano de Pragas na América Latina.(257-277). Piracicaba: FEALQ.

Braga GUL, Rangel, DRN, Flint SD, Anderson AJ, Roberts DW (2006). Conidial pigmentation is important to tolerance against solar-simulated radiation in the entomopathogenic fungus *Metarhizium anisopliae*. Photochem. Photobiol. 8:418-422.

Chelico L, Khachatourians GG (2008). Isolation and characterization of nucleotide excision repair deficient mutants of the entomopathogenic fungus *Beauveria bassiana*. J. Invertebr. Pathol. 98:93-100.

Elzein A, Kroschel J, Cadisch G (2008). Efficacy of Pesta granular formulation of *Striga*-mycoherbicide *Fusarium oxysporum* f. sp. *strigae* Foxy 2 after 5-year of storage. J. Plant Dis. Protect. 115:259-262.

Francisco EA, Mochi DA, Correia ACB, Monteiro AC (2006). Influence of culture media in viability test of conidia of entomopathogenic fungi. Ciência Rural 36:1309-1312.

Francisco EA, Rangel DEN, La Scala Jr N, Barbosa JC, Correia ACB (2008). Exposure of *Metarhizium anisopliae* conidia to UV-B radiation reduces its virulence. J. Anhui Agric. Univ. 35:246-249.

Fravel DR, Connick WJ, Lewis JA (1998). Formulation of microorganis to control plant diseases. *In*: Burges HD (ed.) Formulation of microbial biopesticides: beneficial microorganisms, nematodes and seed treatments (p.187-202). Dordrecht: Kluwer Academic Publishers.

Gaugler R. (1997). Alternative paradigms for commercializing biopesticides. Phytoparasitica 25:179-182.

Lim HW, Thomas L, Rigel DS (2005). Photoprotection. In: Rigel DS, Weiss RA, Lim HW, Dover JS (eds) Photoaging (p.397). Marcel Dekker, Inc..

Machado ACR, Mochi, DA, Monteiro AC (2013). Crop optimization and pre-steps standardization to get a *Bipolaris euphorbiae*-based bioherbicide. Pesqui. Agropecu. Trop. 43:392-399.

Moraes C, Monteiro AC, Mochi DA (2011). Reaction of *Bipolaris euphorbiae* to environmental factors and its tolerance to solar and ultraviolet radiation. World J. Microb. Biotechnol. 27(6):1449-1456.

Moraes C, Monteiro AC, Machado ACR, Barbosa JC, Mochi DA (2014). Production of a bioherbicide agent in liquid and solid medium and in a biphasic cultivation system. Planta Daninha 32:255-264.

Morentini A, Melo IS (2007). Formulação do fungo *Coniothyrium minitans* para controle do mofo-branco causado por *Sclerotinia sclerotiorum*. Pesq. Agropec. Bras 42:155-161.

Penariol MC, Monteiro AC, Pitelli RA, Pereira GT (2008a). Produção de *Bipolaris euphorbiae* em meios de cultura sólidos e líquidos obtidos de grãos e resíduos agroindustriais. Bragantia 67:805-814.

Penariol MC, Monteiro AC, Pitelli RA (2008b). Crescimento e esporulação de *Bipolaris euphorbiae* cultivado sob diferentes condições nutricionais. Cienc. Rural 38:1907-1913.

Rangel DE, Anderson AJ, Roberts DW (2006). Growth of *Metarhizium anisopliae* on non-preferred carbon sources yields conidia with increased UV-B tolerance. J. Invertebr. Pathol. 93:127-134.

Rangel DE, Braga GU, Anderson AJ, Roberts DW (2005). Influence of growth environment on tolerance to UVB radiation, germination speed, and morphology of *Metarhizium anisopliae var. acridum* conidia. J. Invertebr. Pathol. 90:55-58.

Rangel DE, Braga GUL, Fint SD, Anderson AJ, Roberts DW (2004). Variations in UV-B tolerance and germination speed of *Metarhizium anisopliae* conidia produced on insects and artificial substrates. J. Invertebr. Pathol. 87:77-83.

Rossi-Zalaf LS, Alves SB, Lopes RB, Silveira Neto S, Tanzini MR (2008). Interação de microrganismos com outros agentes de controle de pragas e doenças. In: Alves SB, Lopes R B (eds.), Controle microbiano de pragas na América Latina: avanços e desafio*s* (270-302)**.** Piracicaba, SP: FEALQ.

Santos PS, Silva MAQ, Monteiro AC, Gava CAT (2011). Improving photoprotection of *Beauveria bassiana* conidia for biological control of the cactus pest *Dactylopius opuntiae* in the semiarid region northeast of Brazil. Biocontrol Sci Techn. 21:893-902.

Tessmann DJ (2011). Controle biológico: aplicações na área de ciências das plantas daninhas. In: Oliveira Jr RS, Constantin J, Inoue MH (eds) Biologia e manejo de plantas daninhas.Curitiba, PR: Omnipax.

Weikert-Oliveira RCB, de Resende MA, Valerio HM, Caligiorne RB, Paiva E (2002). Genetic variation among pathogens causing "*Helminthosporium*" diseases of rice, maize and wheat. Fitopatol Bras 27:639-643.

Wissing SA, Muller H (2001). Solid lipid nanoparticles (SLN) - a novel carrier for UV lockers. *Pharmazie* 56:783-786.

Wyss GS, Charudattan R, Rosskopf EN, Pitelli RC (2004). Effects of selected pesticides and adjuvants on germination and vegetative growth of *Phomopsis amaranthicola*, a biocontrol agent for *Amaranthus spp*. Weed Res. 44:469-482.

academicJournals

Vol. 10(43), pp. 1837-1843, 21 November, 2016
DOI: 10.5897/AJMR2016.8227
Article Number: 6AD7FFF61667
ISSN 1996-0808
Copyright © 2016
Author(s) retain the copyright of this article
http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Full Length Research Paper

Identification and tracking of microorganisms from the biofilms of container walls used for water storage: Case of rural communities in Burkina Faso

Seyram K. Sossou[1]*, Franck Lalanne[1], Mariam Dakoure/Sou[1] and Naoyuki Funamizu[2]

[1]Laboratory of Water, Sanitation, Ecosystems and Health (LEDES), International Institute for Water and Environmental Engineering (2iE), PO Box 594, 01 Rue de la science, Ouagadougou, Burkina Faso.
[2]Department of Environmental Engineering, University of Hokkaido, Kita 13-Nishi 8, Kita-ku, Sapporo, Hokkaido 060–8628, Japan.

Received 22 July 2016; Accepted 21 September, 2016.

Safe drinking water is an important necessity for humans. In rural communities of Burkina Faso, many households use local containers for storing drinking water. During water storage, some microorganisms get attached to the surface walls of the containers to form a biofilm which can deteriorate drinking water quality over time. This study aimed at evaluating the attachment of indicator microorganisms to containers walls during drinking water storage. Raw drinking water from wells and drilling were stored in three different containers: earthenware jar, polyethylene and galvanized steel for a period of 48 h. During the experiment, attached total coliform, *Escherichia coli*, Enterococci, *Clostridium perfringens*, somatic and F-specific coliphages were measured according to standard methods. Bacteria were enumerated by using conventional membrane filtration procedure and coliphages were done using double layer plaque assays. The results showed that, the adhesion of indicator microorganisms on the surface of earthenware jar, polyethylene and galvanized steel containers was detected and this adhesion was correlated to different parameters, such as temperature, pH, turbidity, concentration of organic nutrients and indigenous microorganism communities. The survival and regrowth of indicator microorganisms on the container walls was due to the quality of raw drinking water before storage. Clay-based material was subjected to more attachment of indicator microorganisms than that of plastic-based polyethylene and metal-based galvanized steel. The lowest yield of biofilm formation by indicator microorganisms was *Clostridium perfringens* (<1 cfu.cm^{-2}) while the highest was total coliform (355 cfu.cm^{-2}). However, the persistence of indicator microorganisms on container walls during drinking water storage deteriorates the water quality more. To meet national guidelines of drinking water quality, it is important to conduct simple water treatment regime such as chlorination before and during water storage in containers.

Key words: Attachment, indicator microorganisms, biofilm, water containers, drinking water, rural communities.

INTRODUCTION

In the next decades, global population growth will place an increasing pressure on the environment, and thereby threaten key resources, such as water. Effects of the current water crisis are already noticeable: more than a

billion of people have no access to safe drinking water (WHO/UNICEF, 2015). As a result, millions of people, mostly young children die every year from water-related diseases, mainly in lower- and middle-income countries. Water-borne diseases are any illness caused by drinking water contaminated by human or animal feces, which contained pathogenic microorganisms (Ashbolt, 2015). These diseases are usually infectious diarrhea such as cholera and typhoid among others. Whilst the detection of microbial pathogens has largely improved, several waterborne microorganisms may persist in so-called "safe" drinking water (Richards et al., 2015). Therefore, the issue of water quality in the transmission of diseases in humans remains a matter of great concern.

In Burkina Faso, 21% of the population has no access to adequate supply and potable water, especially in rural area, where most communities use raw groundwaters for drinking (WHO/UNICEF, 2015). Populations in rural environment are confronted with the optimal management of water supply points (Boubacar et al., 2013).

To manage the increase in water demand, measures undertaken generally focused on the quantitative aspect to meet the needs of the populations. However, the issue of water quality, responsible for diarrheal disease and other diseases associated with microorganisms are the leading cause of infant mortality in Burkina Faso (Some et al., 2014). Thus, the quality of water consumed by rural populations in Burkina Faso is a concern because of the traditional water sources competition, the lack of maintenance of hydraulic structures and the lack of appropriate disinfection methods at house level (Dianou et al., 2011).

In Burkina Faso, water from wells and drilling as ground-water sources are still used and are remained the major drinking water in rural areas for human consumption (Boubacar et al., 2013). From these water source points, the water is only provided at a certain time interval during the day. Although, connected to a supply system, the user still has to store water to have a sufficient amount available for non-supply periods. In addition, because of the long distance between drinking water sources and households, containers are used for the storage of drinking water. Water storage is therefore a necessity both for those who are connected to a non-continuous water supply system and those who depend on drinking water sources (Günther and Schipper, 2013). Under hot climatic conditions in Burkina Faso, households usually store water in local containers: earthenware jar, polyethylene bucket and galvanized steel. During the storage of water in the containers, it is well known that some microorganisms can get attached to the surface walls of the containers and form a biofilm (Hambsch et al., 2013). Attached bacteria can detach from the surface walls and this leads to continuous recontamination of the water (Mathieu et al., 2014). Besides, there is a problem of bacterial regrowth which is related to the increase in bacteria contained in the water (Ikonen et al., 2013). Factors known to affect recontamination of water during storage at home are size of the storage vessel mouth, transfer of water between containers from collection to storage, hand contact and dipping of utensils (Singh et al., 2013), but also bacterial regrowth within the storage container (Machdar et al., 2013) and prospering of organisms in biofilms of containers (Ahmed et al., 2013). A large variety of different heterotrophic bacteria from pathogenic to non-pathogenic ones have been isolated from drinking water biofilm (Richards et al., 2015). So, during storage, water can be deteriorated to a quality often not safe for human consumption.

As a result of the increasing number of households using these containers for storing drinking water in rural areas, it is essential to assess the impact of biofilm formation on water quality to prevent the microbial survival and regrowth in water. Moreover, the type of container material may considerably influence the density of biofilm formation (Waines et al., 2011). Momba and Kaleni (2002) conducted a similar study based on plastic and metal material, but the storage containers made with clay material have not been investigated. Therefore, this study aimed at evaluating the attachment of indicator microorganisms to different water containers used in household for storage of drinking water by rural communities in Burkina Faso.

MATERIALS AND METHODS

Study area

The study covered a rural community named "Ziniare" village (250 000 habitants) which is 35 km from Ouagadougou, the capital city of Burkina Faso. Their main occupation is agriculture and their income is from crops selling. The lack and/or inadequacy of drinking water remained the main problem faced by the inhabitants. Therefore, the rural community of "Ziniare" uses well water and drilling water (boreholes) for drinking in their households. This study was realized focused because of the failure of several project for water supply and particular water quality. A questionnaire related to the type of containers and the duration for water storage was administered to 50 households of the village.

Filling of containers and sampling of stored waters

Two pilot families were chosen for this experiment. These two families were selected as representative of a family in "Ziniare". Each family constituted of a father, mother and three children and having access to both well water and drilling water. During three

*Corresponding author. E-mail : seyram.sossou@2ie-edu.org. Tel: +22671923148.

Author(s) agree that this article remains permanently open access under the terms of the Creative Commons Attribution License 4.0 International License

Table 1. Physico-chemical quality of raw waters from well and drilling.

Parameter	Well water			Drilling water		
	EJ	PE	GS	EJ	PE	GS
Temperature (°C)	26.5±1.15	30.1±1.45	30.6±1.22	25.4±1.26	29.1±1.31	29.8±1.39
pH	7.57±0.16	7.59±0.16	7.55±0.16	7.18±0.16	7.21±0.16	7.17±0.27
Turbidity (NTU)	32.01±4.46	27.60±3.67	33.24±5.21	9.24±1.73	9.32±1.26	9.19±1.02
DOC (mgl^{-1})	21.54±2.91	17.69±3.01	14.73±2.72	19.35±2.23	15.18±1.98	12.64±1.69

weeks, raw waters (12 samples) for the experiment were collected once a week from well and drilling in the two households. After cleaning with soap water, the earthenware jar (EJ), polyethylene (PE) and galvanized steel (GS) were filled with 25 L of tested waters and transported to the laboratory to be store at ambient temperature as the community normally does (without any disinfection and covered with a lid). A 25 L sample drinking water was collected as control and stored in a sterile bottle at 4°C.

Physico-chemical analysis

Temperature and pH were measured using a hand-held multi-parameter Hanna Instruments (HI-98129, Inc., Woonsocket, Rhode Island, USA). Turbidity was measured using a hand-held turbidity meter Hanna Instruments (HI-93102, Keysborough, Australia). The glassware receiving the water samples for the dissolved organic carbon (DOC) analysis was muffled at 500°C for 4 h after cleaning. DOC concentrations were measured using a total organic carbon analyzer (Dohrmann DC-180, Sigma-Aldrich, Belgium) which uses UV-promoted persulfate oxidation. Samples were previously filtered on carbon free borosilicate 0.7 µm pore-size filter to remove particulate organic carbon.

Detachment of microorganisms from EJ, PE and GS container walls

The PE and GS containers were bought at the local market and have the shape of a bucket. The EJ containers were made especially for the experiment by local manufacturer and also have the shape of a bucket. For the detachment of microorganisms from EJ, PE and GS surfaces, some slides were considered. Therefore, 8 slides per sampling were aseptically removed from each container after 24 and 48 h and transferred into sterile plastic bottles containing 100 ml saline. To release attached microorganisms into the saline, the contents were mixed for 5 min using a vortex mixer.

Microorganism's enumeration

Indicator bacteria were enumerated by using conventional membrane filtration procedure according to International Standards Organization (ISO) protocols. The ISO 9308-1 (2000), ISO 7899-2 (2000) and ISO 17994 (2014) were used for the detection of total coliforms, *E. coli,* Enterococci and *Clostridium perfringens*, respectively. Water samples, of 100 ml were filtered through hydrophilic mixed cellulose esters membranes (Pall Corporation) of 0.45 µm pore size and 47 mm diameter. Sterile Petri dishes were filled with selective media: chromocult coliform agar ES (Merck, Germany) was used for simultaneous enumeration of total coliform and *E. coli*, chromocult Enterococci agar (Merck, Germany) was used for Enterococci and tryptose sulphite cycloserine agar (Difco, Detroit USA) was used for *C. perfringens*. After filtration, membranes were placed in each Petri dish and incubated at 37°C for 24 h, at 44.5°C for 24 h, at 37°C for 48 h and 44°C for 24 to 48 h, for each media and temperature, respectively.

The detection of F-specific and somatic coliphages was carried using double layer plaque assays according to the ISO 10705-1 (1995) and ISO 10705-2 (2000) standards, respectively. The host bacterial strains WG49 and WG5 were exposed to the eluates to culture the F-specific and somatic coliphages, respectively. In the presence of both F-specific and somatic coliphages, plaques could be enumerated after overnight culture. All analyses were done in triplicate. The following equations (1 and 2) were used to calculate the number of attached bacteria and coliphages, respectively:

$$\text{cfu cm}^{-2} = \text{ND/surface area of slides} \quad (1)$$

$$\text{pfu cm}^{-2} = \text{ND/surface area of slides} \quad (2)$$

where N is the number of microorganisms, D the dilution factor.

RESULTS

Results of investigations

For the collection of drinking water, the results showed that 66, 21 and 13% of households use EJ, PE and GS containers, respectively. It was also revealed that 58% of households stored their water for 24 to 48 h, 26% for 12 to 24 h, 14% for 6 to 12h and 2% for about three days. Therefore, this study selected an average storage period of 48 h as the largest percentage (58%) of households which store their water for that period of time.

Physico-chemical quality of raw waters from well and drilling

The physico-chemical quality of raw waters from well and drilling is presented in Table 1. The temperature in all containers ranged from 26.5 to 30.6°C, which was relatively close to the annual mean of ambient temperature in Burkina Faso. Neutral pH range of 7.17 to 7.59 was recorded in all the containers. The turbidity ranged from 9.19 to 33.24 NTU and therefore did not meet the turbidity limit (<5 NTU) acceptable in Burkina Faso/WHO (2005) guidelines. The DOC ranged from 12.64 to 21.54 mgl^{-1} and showed a significant presence of organic nutrients.

Table 2. Microbiological quality of raw waters from well and drilling.

Parameters	Well water (n=30)		Drilling water (n=30)	
	Ranges	Average	Ranges	Average
Total coliforms (cfu/100 ml)	996-3.19x10^3	2.98x10^3	76-1.98x10^3	1.41x10^3
Esherichia coli (cfu/100 ml)	54-135	84	<1-62	23
Clostridium perfringens (cfu/100 ml)	<1-27	11	<1-6	<1
Somatic coliphage (pfu/100 ml)	<1-650	432	<1-206	74
F-specific coliphage (pfu/100 ml)	<1-145	107	<1-35	19

Table 3. Growth of indicator microorganisms on the surface of containers during drinking water storage.

Parameters	Well water (n=30)						Drilling water (n=30)					
	EJ		PE		GS		EJ		PE		GS	
	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h
Total coliforms (cfu.cm^{-2})	266	355	105	174	37	51	75	124	54	88	7	11
Escherichia coli (cfu.cm^{-2})	9	5	5	2	4	3	7	11	4	5	2	3
Clostridium perfringens (cfu.cm^{-2})	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1
Somatic coliphage (pfu.cm^{-2})	7	9	4	6	1	2	11	13	7	9	5	8
F-specific coliphage (pfu.cm^{-2})	2	5	1	3	2	3	8	12	5	7	2	5

Microbiological quality of raw waters from well and drilling

The microbiological quality of raw waters from well and drilling is presented in Table 2. Raw waters from well and drilling were contaminated by total coliforms (an average of 2.98 × 10^3 and 1.41 × 10^3 cfu/100ml, respectively), *E. coli* (an average of 84 and 23 cfu/100ml, respectively), *C. perfringens* (an average of 11 and <1 cfu/100ml, respectively), somatic coliphage (an average of 432 and 74 pfu/100 ml, respectively) and F-specific coliphage (average of 107 and 19 pfu/100 ml, respectively). The Burkina Faso/WHO (2005) guidelines for water quality required the absence of coliform bacteria in water. Therefore, the raw waters collected for this experimental study were not safe for drinking.

Growth of indicator microorganisms on the container walls during storage of drinking water

The growth of indicator microorganisms on the container walls during the storage of drinking water is presented in Table 3. Attached indicator microorganisms were observed during the experiment period. The yield (average count) of total coliforms, *E. coli*, *C. perfringens,* somatic coliphage and F-specific coliphage in all containers during the study period (48 h) was 7 to 355, 2 to 11, < 1, 8-13 and 3-12 pfu cm^{-2}, respectively. The lowest yield of indicator microorganisms was noted for *C. perfringens*, while the highest yield was noted for total coliforms. Moreover, the occurrence and survival of total coliforms was greater on EJ container walls than PE and GS container walls. The regrowth of indicator microorganisms in all the containers occurred 48 h after their exposure to stored waters.

DISCUSSION

The experimental protocol described in this study was designed to evaluate the attachment of indicator microorganisms to container walls used in household for drinking water by rural communities in Burkina Faso. As a result, the adhesion of indicator microorganisms on the surface of EJ, PE and GS containers, was detected and cause formation of biofilms. Studies of Strathmann et al. (2013) showed that in water storage systems, microorganisms are mainly present on internal surfaces as attached bacteria as a biofilm, with a minor part in the water phase. Biofilm formation during water storage in this study is related to temperature, pH, turbidity, concentration of organic nutrients and indigenous microorganism's community of the stored water. Indeed, biofilm formation in water distribution systems depends on a variety of factors including the physico-chemical properties of water, the composition of biofilms, factors governing their formation and the effect and significance of these biofilms (Wu et al., 2015).

The adhesion of indicator microorganisms on the surface of water storage containers is supported by the level of temperature detected in the drinking water during storage. The ability of bacteria to grow and form a biofilm over a wide range of temperature has been studied.

Silhan et al. (2006) have shown that biofilm formation in drinking water systems were denser at about 35°C than at about 15°C. In this study, temperatures ranging from 26.5 to 30.6°C were recorded in all containers, creating favorable microorganism growing conditions. Therefore, for Strathmann et al. (2013), temperature is considered to be an important regulator of biofilm growth, especially in non-disinfected water. At the same time, during water storage, neutral pH was obtained in the different containers investigated. It is well known that neutral pH condition contributes considerably to increase in the formation of biofilm (Jones et al., 2015).

The turbidity level in different containers showed the presence of a concentration of suspended solids. It has been revealed that, there is a link between high turbidity level and the growth of microorganisms in water because the turbidity can serve as a source of nutrients for waterborne bacteria, protozoa and viruses (Wingender and Flemming, 2011). At the same time, the concentration of organic nutrients (DOC content) could be responsible for the microbial growth potential enabling the buildup of microorganisms on storage containers walls. The link between organic content and bacterial growth resulting in biological aggregates that may attach to the surface of distribution systems has been reported (Liu et al., 2013). Biofilm formation is usually promoted on the surface of a material if that material is able to supply the required nutrients for microbial growth (Strathmann et al., 2013). Therefore, the occurrence and persistence of micro-organisms on the surface of EJ, PE and GS could be related to the level of turbidity and DOC in stored waters.

One of the other important factors involved in the attachment of indicator microorganisms is their occurrence and their persistence in stored waters. This is due to the original presence of indicator microorganisms in the raw waters (from well and drilling). The survival and regrowth of indicator microorganisms during biofilm formation is due to the occurrence of indigenous microorganism's community in the raw waters (Wu et al., 2015). As total coliform and *E. coli* were constantly present in raw waters, they were also found in stored waters in the different containers. However, only *C. perfringens* was recorded in stored waters at a low concentration during the study period. Concerning the coliphages, a higher count was found in the well water than in the drilling water. With the exception of *C. perfringens*, the survival of indicator microorganisms on the surface of household containers consisted of total coliform, and *E. coli*, somatic and F-specific coliphages, although the yield for coliphages was lower than that of total coliforms and *E. coli*. Whereas the occurrence and persistence of total coliforms and *E. coli* was always found during storage on the surface of containers, other microorganisms not investigated in this study, could occur. So competition of microorganism's community for limited organic nutrients could be responsible for the DOC concentration in the stored waters.

This study has shown that the regrowth of indicator microorganisms occurred 48 h after the exposure of water in containers. This regrowth was observed more with total coliforms than with other indicator micro-organisms studied. It is well known that total coliforms include a heterogeneous group constituting the genera *Escherichia, Citrobacter, Klebsiella, Enterobacter, Serratia* and *Rahnella* (Richards et al., 2015). Although, these genera were not identified in this study, they could be released into the stored water and deteriorate its quality. Indeed, biofilm formation in drinking water systems may deteriorate water quality by microbial activity, and in some cases, constitute a serious health hazard for consumers, either due to pathogenic bacteria growing in the biofilm or since biofilms may provide a safe haven for intruding pathogens (Fabris et al., 2016). Moreover, the persistence of coliphages on the surface of storage containers was observed and their potential of regrowth was even minimized during the storage. However, their release into drinking water could lead to a risk of viral infection for consumers.

During water storage, clay-based material (EJ) supported more attachment of indicator microorganisms than the plastic-based material (PE) and the metal-based material (GS). So, there is a direct relationship between the type of surface of storage materials and the density of biofilm formation. Several investigations have shown significant differences in biofilm formation on various materials over several months (Waines et al., 2011), showing that container material influences considerably biofilm formation, at least, in the short term. Thus, the quality of water during storage depends on the type of surface of storage materials. In the present study, water quality is more affected in EJ than PE and GS. The study revealed that the level of DOC was higher in EJ-stored water than in PE-stored and GS-stored ones. This could also explain an increase in total coliform numbers on the surface of EJ than on PE and GS containers.

The results of this study has revealed that indicator microorganisms have survived and regrown in EJ, PE and GS containers during water storage with biofilm formation. This formation of biofilm on the surface of containers could deteriorate the quality of the water stored. Therefore, a suitable education and information program related to water safety and hygiene practices should be provided for rural communities in order to minimize microorganism's regrowth in water stored in containers. In this regard, it is also important to previously perform a disinfection of the drinking water before storage.

Conclusion

This study investigated the attachment of indicator micro-organisms to water storage containers used in household drinking water by rural communities in Burkina Faso. The

results showed that, indicator microorganisms (total coliform, *E. coli*, Enterococci, *C. perfringens*, somatic and F-specific coliphages) were attached on the inner surface of EJ, PE and GS containers and this was linked to different parameters such as temperature, pH, turbidity, concentration of organic nutrients and indigenous microorganism's community. The survival and regrowth of indicator microorganisms on the inner surface of containers is due to the bad quality of raw waters before storage. Clay-based material (EJ) supported more attachment of indicator microorganisms than that of plastic-based material (PE) and metal-based material (GS). The lowest yield of biofilm formation by the indicator microorganisms was noted for *C. perfringens* while the highest was noted for total coliform. The persistence of indicator microorganisms on the surface of water containers could deteriorate the quality of drinking water and therefore, it is important to adopt simple water treatment regime such as chlorination before and during water storage in containers.

Competing interests

The authors declare that there is no conflict of interest.

ACKNOWLEDGEMENTS

The authors wish to express their sincere thanks to the staff of the Laboratory of Water, Sanitation, Ecosystem and Health (LEDES), for the technical assistance rendered during the course of the work.

REFERENCES

Ahmed D, Islam MS, Begum YA, Janzon A, Qadri F, Sjöling Å (2013). Presence of enterotoxigenic *Escherichia coli* in biofilms formed in water containers in poor households coincides with epidemic seasons in Dhaka. J. Appl. Microbiol. 114(4):1223-1229.

Ashbolt NJ, (2015). Microbial contamination of drinking water and human health from community water systems. Curr. Environ. Health Rep. 2: 95-106.

Boubacar S, Aminata K, Dramane Z, Noel PJ, Hortense B, Francis R, Dianou D (2013). Problematic of Drinking Water Access in Rural Area: Case Study of the Sourou Valley in Burkina Faso. J. Environ. Prot. 4: 31-50.

Burkina Faso/WHO (2005). National drinking water quality guidelines. Joint Decision n° 0019/MAHRH/MS (Ministry of Agriculture, Water Resources and Fisheries/ Ministry of Health).

Dianou D, Savadogo B, Zongo D, Zougouri T, Poda JN, Bado H, Rosillon F (2011). Surface Waters Quality of the Sourou Valley: The Case of Mouhoun, Sourou, Debe and Gana Rivers in Burkina Faso [In french]. Int. J. Inn. Biol. Chem. Sci. 5(4):1571-1589.

Fabris R, Braun K, Ho L, Verberk JQJC, Drikas M (2016). Bacteriological water quality changes in parallel pilot distribution systems. Water Sci. Technol. Water Supply, ws2016095. Günther I, Schipper Y (2013). Pumps, germs and storage: the impact of improved water containers on water quality and health. Health Econ. 22(7):757-774.

Hambsch B, Ashworth J, van der Kooij D (2013). Enhancement of microbial growth by materials in contact with drinking water: problems and test methods. In: D van der Kooij, PWJJ van der Wielen (eds) Microbial growth in drinking water supplies. Problems, causes, control and research needs. London: IWA Publishing 12:339-361.

Ikonen J, Pitkänen T, Miettinen IT (2013). Suitability of optical, physical and chemical measurements for detection of changes in bacterial drinking water quality. Int. J. Environ. Res. Public Health. 10(11):5349-5363.

ISO 10705-1 (1995). Water quality Detection and enumeration of bacteriophages. Part 1: enumeration of F-specific RNA coliphages. International Organization for Standarization, Geneva, Switzerland, 11p. Consulted September 2016.

ISO 10705-2 (2000). Water quality-detection and enumeration of bacteriophages. Part 2. Enumeration of somatic coliphages. International Organization for Standarization, Geneva, Switzerland, 16p. Consulted September 2016.

ISO 17994 (2014). Water Quality - Requirements for the Comparison of the Relative Recovery of Microorganisms by Two Quantitative Methods. International Organization for Standardization. Geneva, Switzerland, 22p. Consulted September 2016.

ISO 7899-2 (2000). Water quality - detection and enumeration of intestinal enterococci – Part 2: Membrane filtration method. International Organization for Standardization. Geneva, Switzerland, 7p.

ISO 9308-1 (2000). Water quality – detection and enumeration of *Escherichia coli* and coliform bacteria - Part 1: Membrane filtration method. International Organization for Standardization. Geneva, Switzerland, 10p.

Jones EM, Cochrane CA, Percival SL (2015). The Effect of pH on the Extracellular Matrix and Biofilms. Adv. Wound. Care. 4(7): 431-439.

Liu G, Lut MC, Verberk JQJC, Van Dijk JC (2013). A comparison of additional treatment processes to limit particle accumulation and microbial growth during drinking water distribution. Water Res. 47(8):2719-2728.

Machdar E, van der Steen NP, Raschid-Sally L, Lens PNL (2013). Application of quantitative microbial risk assessment to analyze the public health risk from poor drinking water quality in a low income area in Accra, Ghana. Sci. Total Environ. 449:134-142.

Mathieu L, Bertrand I, Abe Y, Angel E, Block JC, Skali-Lami S, Francius G (2014). Drinking water biofilm cohesiveness changes under chlorination or hydrodynamic stress. Water Res. 55:175-184.

Momba MN, Kaleni P (2002). Regrowth and survival of indicator microorganisms on the surfaces of household containers used for the storage of drinking water in rural communities of South Africa. Water Res. 36(12):3023-3028.

Richards CL, Broadaway SC, Eggers MJ, Doyle J, Pyle BH, Camper AK, Ford TE (2015). Detection of pathogenic and non-pathogenic bacteria in drinking water and associated biofilms on the crow reservation, Montana, USA. Microb. Ecol. 1-12.

Silhan J, Corfitzen C, Albrechtsen H (2006). Effect of temperature and pipe material on biofilm formation and survival of Escherichia coli in used drinking water pipes: a laboratory-based study. Water Sci. Technol. 54(3):49-56.

Singh U, Lutchmanariyan R, Wright J, Knight S, Jackson S, Langmark J, Vosloo D, Rodda N (2013). Microbial quality of drinking water from groundtanks and tankers at source and point-of-use in eThekwini Municipality, South Africa, and its relationship to health outcomes. Water SA. 39(5):663-673.

Some YSC, Soro TD, Ouedraogo S (2014). Study of the prevalence of diseases related to water and influences of environmental factors in the district of Nomgr-Masson: case Tanghin area (Ouagadougou, Burkina Faso) [In french]. Int. J. Inn. Biol. Chem. Sci. 8(1):289-303.

Strathmann M, Mittenzwey KH, Sinn G, Papadakis W, Flemming HC (2013). Simultaneous monitoring of biofilm growth, microbial activity, and inorganic deposits on surfaces with an in situ, online, real-time, non-destructive, optical sensor. Biofouling 29(5):573-583.

Waines PL, Moate R, Moody AJ, Allen M, Bradley G (2011). The effect of material choice on biofilm formation in a model warm water distribution system. Biofouling. 27(10):1161-1174.

WHO and UNICEF (2015) Progress on Sanitation and Drinking-Water. 2015 update. WHO Press, Geneva, Switzerland.

Wingender J, Flemming HC (2011) Biofilms in drinking water and theirrole as reservoir for pathogens. Int. J. Hyg. Environ. Health.

214(6):417-423.
Wu H, Zhang J, Mi Z, Xie S, Chen C, Zhang X (2015). Biofilm bacterial communities in urban drinking water distribution systems transporting waters with different purification strategies. Appl. Microbiol. Biotechnol. 99(4):1947-1955.

Related Journals Published by Academic Journals

- *African Journal of Biotechnology*
- *African Journal of Biochemistry Research*
- *Journal of Bacteriology Research*
- *Journal of Evolutionary Biology Research*
- *Journal of Yeast and Fungal Research*
- *Journal of Brewing and Distilling*

academicJournals