ABOUT AJMR

The African Journal of Microbiology Research (AJMR) (ISSN 1996-0808) is published Weekly (one volume per year) by Academic Journals.

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.

Submission of Manuscript

Please read the Instructions for Authors before submitting your manuscript. The manuscript files should be given the last name of the first author

Click here to Submit manuscripts online

If you have any difficulty using the online submission system, kindly submit via this email ajmr@academicjournals.org.

With questions or concerns, please contact the Editorial Office at ajmr@academicjournals.org.
Editors

Prof. Dr. Stefan Schmidt,
Applied and Environmental Microbiology
School of Biochemistry, Genetics and Microbiology
University of KwaZulu-Natal
Private Bag X01
Scottsville, Pietermaritzburg 3209
South Africa.

Prof. Fukai Bao
Department of Microbiology and Immunology
Kunming Medical University
Kunming 650031,
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,
P.O.B. 12272, IL-91120 Jerusalem,
Israel

Prof. Long-Liu Lin
National Chiayi University
300 Syuefu Road,
Chiayi,
Taiwan

N. John Tonukari, Ph.D
Department of Biochemistry
Delta State University
PMB 1
Abraka, Nigeria

Dr. Thaddeus Ezeji
Assistant Professor
Fermentation and Biotechnology Unit
Department of Animal Sciences
The Ohio State University
1680 Madison Avenue
USA.

Associate Editors

Dr. Mamadou Gueye
MIRCEN/ Laboratoire commun de microbiologie
IRD-ISRA-UCAD, BP 1386,
DAKAR, Senegal.

Dr. Caroline Mary Knox
Department of Biochemistry, Microbiology and
Biotechnology
Rhodes University
Grahamstown 6140
South Africa.

Dr. Hesham Elsayed Mostafa
Genetic Engineering and Biotechnology Research
Institute (GEBRI)
Mubarak City For Scientific Research,
Research Area, New Borg El-Arab City,
Post Code 21934, Alexandria, Egypt.

Dr. Wael Abbas El-Naggar
Head of Microbiology Department,
Faculty of Pharmacy,
Mansoura University,
Mansoura 35516, Egypt.

Dr. Abdel Nasser A. El-Moghazy
Microbiology, Molecular Biology, Genetics Engineering
and Biotechnology
Dept of Microbiology and Immunology
Faculty of Pharmacy
Al-Azhar University
Nasr city,
Cairo, Egypt
Editorial Board

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

Dr. Rachna Chandra
Environmental Impact Assessment Division
Environmental Sciences
Sálim Ali Center for Ornithology and Natural History (SACON), Anaikatty (PO), Coimbatore-641108, India

Dr. Yongxu Sun
Department of Medicinal Chemistry and Biomacromolecules
Qiqihar Medical University, Qiqihar 161006 Heilongjiang Province
P.R. China

Dr. Ramesh Chand Kasana
Institute of Himalayan Bioresource Technology
Palampur, Distt. Kangra (HP), India

Dr. S. Meena Kumari
Department of Biosciences
Faculty of Science
University of Mauritius
Reduit

Dr. T. Ramesh
Assistant Professor
Marine Microbiology
CAS in Marine Biology
Faculty of Marine Sciences
Annamalai University
Parangipettai - 608 502
Cuddalore Dist. Tamilnadu, India

Dr. Pagano Marcela Claudia
Post doctoral fellowship at Department of Biology, Federal University of Ceará - UFC, Brazil.
Dr. EL-Sayed E. Habib  
Associate Professor, 
Dept. of Microbiology, 
Faculty of Pharmacy, 
Mansoura University, 
Egypt.

Dr. Pongsak Rattanachaikunsopon  
Department of Biological Science, 
Faculty of Science, 
Ubon Ratchathani University, 
Warin Chamrap, Ubon Ratchathani 34190, 
Thailand

Dr. Gokul Shankar Sabesan  
Microbiology Unit, Faculty of Medicine, 
AIMST University, 
Jalan Bedong, Semeling 08100, 
Kedah, 
Malaysia

Dr. Kwang Young Song  
Department of Biological Engineering, 
School of Biological and Chemical Engineering, 
Yanbian University of Science and Technology, 
Yanji, 
China.

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

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

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

Dr. R. Balaji Raja M.Tech (Ph.D)  
Assistant Professor, 
Department of Biotechnology, 
School of Bioengineering, 
SRM University, 
Chennai, 
India

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

Dr. Mohd Fuat ABD Razak  
Institute for Medical Research 
Malaysia

Dr. Davide Pacifico  
Istituto di Virologia Vegetale – CNR 
Italy

Prof. Dr. Akrum Hamdy  
Faculty of Agriculture, Minia University, 
Egypt

Dr. Ntobeko A. B. Ntusi  
Cardiac Clinic, Department of Medicine, 
University of Cape Town and 
Department of Cardiovascular Medicine, 
University of Oxford 
South Africa and 
United Kingdom

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 Svetlana Nikolić  
Faculty of Technology and Metallurgy, 
University of Belgrade, 
Serbia

Dr. Sivakumar Swaminathan  
Department of Agronomy, 
College of Agriculture and Life Sciences, 
Iowa State University, 
Ames, Iowa 50011 
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
Dr. Josephine Nketsia-Tabiri  
Ghana Atomic Energy Commission  
Ghana

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

Dr. Mohammad Nazrul Islam  
NIMR; IPH-Bangalore & NIUM  
Bangladesh

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

Dr. Giuliana Noratto  
Texas A&M University  
USA

Dr. Phanikanth Venkata Turlapati  
Washington State University  
USA

Dr. Khaleel I. Z. Jawasreh  
National Centre for Agricultural Research and Extension, NCARE  
Jordan

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

Dr. S. Meena Kumari  
Department of Biosciences  
Faculty of Science  
University of Mauritius  
Reduit  
Mauritius

Dr. S. Anju  
Department of Biotechnology,  
SRM University, Chennai-603203  
India

Dr. Mustafa Maroufpor  
Iran

Prof. Dong Zhichun  
Professor, Department of Animal Sciences and Veterinary Medicine,  
Yunnan Agriculture University,  
China

Dr. Mehdi Azami  
Parasitology & Mycology Dept,  
Baghaeei Lab.,  
Shams Abadi St.  
Isfahan  
Iran

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,  
USA

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

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

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

Prof. Branislava Kocic  
Specialist of Microbiology and Parasitology  
University of Nis, School of Medicine Institute for Public Health Nis, Bul. Z. Djindjica 50, 18000 Nis  
Serbia

Dr. Rafel Socías  
CITA de Aragón,  
Spain
Prof. Kamal I. Mohamed  
*State University of New York at Oswego, USA*

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

Dr. Mike Agenbag (Michael Hermanus Albertus)  
*Manager Municipal Health Services, Joe Gqabi District Municipality, South Africa*

Dr. D. V. L. Sarada  
*Department of Biotechnology, SRM University, Chennai-603203, India.*

Dr. Samuel K Ameyaw  
*Civista Medical Center, United States of America*

Prof. Huaizhi Wang  
*Institute of Hepatopancreatobiliary Surgery of PLA Southwest Hospital, Third Military Medical University, Chongqing400038, P. R. China*

Prof. Bakhiet AO  
*College of Veterinary Medicine, Sudan University of Science and Technology, Sudan*

Dr. Saba F. Hussain  
*Community, Orthodontics and Pediatric Dentistry Department, Faculty of Dentistry, Universiti Teknologi MARA, 40450 Shah Alam, Selangor, Malaysia*

Prof. Dr. Zohair I.F.Rahemo  
*State Key Lab of Food Science and Technology, Jiangnan University, P. R. China*

Dr. Afework Kassu  
*University of Gondar, Ethiopia*

Prof. Isidro A. T. Savillo  
*ISCOF, Philippines*

Dr. How-Yee Lai  
*Taylor’s University College, Malaysia*

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

Dr. Omotoyin Siyanbola  
*Bowen University, Iwo, Nigeria*

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, MD.  
*Chang Gung Memorial Hospital, Taiwan*

Dr. Liane Raluca Stan  
*University Politehnica of Bucharest, Department of Organic Chemistry “C.Nenitzescu”, Romania*

Dr. Muhamed Osman  
*Senior Lecturer of Pathology & Consultant Immunopathologist, Department of Pathology, Faculty of Medicine, Universiti Teknologi MARA, 40450 Shah Alam, Selangor, Malaysia*

Dr. Mohammad Feizabadi  
*Tehran University of Medical Sciences, Iran*
Prof. Ahmed H Mitwalli  
*State Key Lab of Food Science and Technology*  
*Jiangnan University*  
P. R. China

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

Dr. Ms. Jemimah Gesare Onsare  
*Ministry of Higher, Education Science and Technology*  
Kenya

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

Dr. Ehsan Sari  
*Department of Plan 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 250 Kuo Kuang Rd, Taichung,*  
Taiwan

Dr. Fereshteh Naderi  
*Physical chemist,*  
*Islamic Azad University,*  
*Shahre Ghods Branch*  
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

Prof. Dr. Márcio Garcia Ribeiro (DVM, PhD)  
*School of Veterinary Medicine and Animal Science - UNESP,*  
Dept. Veterinary Hygiene and Public Health,  
State of Sao Paulo  
Brazil

Prof. Dr. 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, PhD  
*Laboratory of Viral Diseases*  
*National Institute of Allergy and Infectious Diseases,*  
*National Institutes of Health*

Dr. Dele Raheem  
*University of Helsinki*  
Finland

Dr. Li Sun  
*PLA Centre for the treatment of infectious diseases,*  
*Tangdu Hospital,*  
*Fourth Military Medical University*  
China
Dr. Biljana Miljkovic-Selimovic
School of Medicine,
University in Nis,
Serbia; Referent laboratory for Campylobacter and Helicobacter,
Center for Microbiology,
Institute for Public Health, 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, 180 Jones Avenue
Rutgers University, New Brunswick, NJ 08901-8536
USA

Dr. Heping Zhang
The Key Laboratory of Dairy Biotechnology and Engineering,
Ministry of Education,
Inner Mongolia Agricultural University.
China

Prof. Natasha Potgieter
University of Venda
South Africa

Dr. Alemzadeh
Sharif University
Iran

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

Dr. Armando Gonzalez-Sanchez
Universidad Autonoma Metropolitana Cuajimalpa
Mexico

Dr. Pradeep Parihar
Lovely Professional University, Phagwara, 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
Laboratory of Bioprospection. University of Brasilia
Brazil

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

Dr. Farid Che Ghazali
Abdelmalek Essaadi University,
Tetouan,
Morocco

Dr. Zainab Z. Ismail
Department of Environmental Engineering, University of Baghdad.
Iraq

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

Dr. Papaevangelou Vassiliki
Athens University Medical School
Greece

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

Dr. Galba Maria de Campos Takaki
Catholic University of Pernambuco
Brazil
<table>
<thead>
<tr>
<th>Name</th>
<th>Institution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dr. Kwabena Ofori-Kwakye</td>
<td>Department of Pharmaceutics, Kwame Nkrumah University of Science &amp; Technology, KUMASI Ghana</td>
</tr>
<tr>
<td>Prof. Dr. Liesel Brenda Gende</td>
<td>Arthropods Laboratory, School of Natural and Exact Sciences, National University of Mar del Plata Buenos Aires, Argentina.</td>
</tr>
<tr>
<td>Dr. Adeshina Gbonjubola</td>
<td>Ahmadu Bello University, Zaria, Nigeria</td>
</tr>
<tr>
<td>Prof. Dr. Stylianos Chatzipanagiotou</td>
<td>University of Athens – Medical School Greece</td>
</tr>
<tr>
<td>Dr. Dongqing BAI</td>
<td>Department of Fishery Science, Tianjin Agricultural College, Tianjin 300384 P. R. China</td>
</tr>
<tr>
<td>Dr. Dingqiang Lu</td>
<td>Nanjing University of Technology P.R. China</td>
</tr>
<tr>
<td>Dr. L. B. Sukla</td>
<td>Scientist –G &amp; Head, Biominerals Department, IMMT, Bhubaneswar India</td>
</tr>
<tr>
<td>Dr. Hakan Parlakpinar</td>
<td>MD. Inonu University, Medical Faculty, Department of Pharmacology, Malatya Turkey</td>
</tr>
<tr>
<td>Dr. Pak-Lam Yu</td>
<td>Massey University New Zealand</td>
</tr>
<tr>
<td>Dr. Percy Chimwamurombe</td>
<td>University of Namibia Namibia</td>
</tr>
<tr>
<td>Dr. Euclésio Simionatto</td>
<td>State University of Mato Grosso do Sul-UEMS Brazil</td>
</tr>
<tr>
<td>Dr. Hans-Jürg Monstein</td>
<td>Clinical Microbiology, Molecular Biology Laboratory, University Hospital, Faculty of Health Sciences, S-581 85 Linköping Sweden</td>
</tr>
<tr>
<td>Dr. Ajith, T. A</td>
<td>Associate Professor Biochemistry, Amala Institute of Medical Sciences, Amala Nagar, Thrissur, Kerala-680 555 India</td>
</tr>
<tr>
<td>Dr. Feng-Chia Hsieh</td>
<td>Biopesticides Division, Taiwan Agricultural Chemicals and Toxic Substances Research Institute, Council of Agriculture Taiwan</td>
</tr>
<tr>
<td>Prof. Dra. Suzan Pantaroto de Vasconcellos</td>
<td>Universidade Federal de São Paulo Rua Prof. Artur Riedel, 275 Jd. Eldorado, Diadema, SP CEP 09972-270 Brasil</td>
</tr>
<tr>
<td>Dr. Maria Leonor Ribeiro Casimiro Lopes Assad</td>
<td>Universidade Federal de São Carlos - Centro de Ciências Agrárias - CCA/UFSCar Departamento de Recursos Naturais e Proteção Ambiental Rodovia Anhanguera, km 174 - SP-330 Araras - São Paulo Brasil</td>
</tr>
<tr>
<td>Dr. Pierangeli G. Vital</td>
<td>Institute of Biology, College of Science, University of the Philippines Philippines</td>
</tr>
<tr>
<td>Dr. Roland Ndip</td>
<td>University of Fort Hare, Alice South Africa</td>
</tr>
<tr>
<td>Dr. Shawn Carraher</td>
<td>University of Fort Hare, Alice South Africa</td>
</tr>
<tr>
<td>Dr. José Eduardo Marques Pessanha</td>
<td>Observatório de Saúde Urbana de Belo Horizonte/Faculdade de Medicina da Universidade Federal de Minas Gerais Brasil</td>
</tr>
<tr>
<td>Name</td>
<td>Institution</td>
</tr>
<tr>
<td>-----------------------</td>
<td>------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>Dr. Yuanshu Qian</td>
<td>Department of Pharmacology, Shantou University Medical College</td>
</tr>
<tr>
<td>Dr. Helen Treichel</td>
<td>URI-Campus de Erechim</td>
</tr>
<tr>
<td>Dr. Xiao-Qing Hu</td>
<td>State Key Lab of Food Science and Technology, Jiangnan University</td>
</tr>
<tr>
<td>Dr. Olli H. Tuovinen</td>
<td>Ohio State University, Columbus, Ohio</td>
</tr>
<tr>
<td>Prof. Stoyan Groudev</td>
<td>University of Mining and Geology “Saint Ivan Rilski”</td>
</tr>
<tr>
<td>Dr. G. Thirumurugan</td>
<td>Research lab, GIET School of Pharmacy, NH-5, Chaitanya nagar, Rajahmundry-533294.</td>
</tr>
<tr>
<td>Dr. Charu Gomber</td>
<td>Thapar University</td>
</tr>
<tr>
<td>Dr. Jan Kuever</td>
<td>Bremen Institute for Materials Testing, Department of Microbiology, Paul-Feller-Str. 1, 28199 Bremen Germany</td>
</tr>
<tr>
<td>Dr. Nicola S. Flanagan</td>
<td>Universidad Javeriana, Cali</td>
</tr>
<tr>
<td>Dr. André Luiz C. M. de A. Santiago</td>
<td>Universidade Federal Rural de Pernambuco</td>
</tr>
<tr>
<td>Dr. Dhruva Kumar Jha</td>
<td>Microbial Ecology Laboratory, Department of Botany, Gauhati University, Guwahati 781 014, Assam</td>
</tr>
<tr>
<td>Dr. N Saleem Basha</td>
<td>M. Pharm (Pharmaceutical Biotechnology), Eritrea (North East Africa)</td>
</tr>
<tr>
<td>Prof. Dr. João Lúcio de Azevedo</td>
<td>Dept. Genetics-University of São Paulo-Faculty of Agriculture- Piracicaba, 13400-970</td>
</tr>
<tr>
<td>Dr. Julia Inés Fariña</td>
<td>PROIMI-CONICET</td>
</tr>
<tr>
<td>Dr. Yutaka Ito</td>
<td>Kyoto University</td>
</tr>
<tr>
<td>Dr. Cheruiyot K. Ronald</td>
<td>Biomedical Laboratory Technologist</td>
</tr>
<tr>
<td>Prof. Dr. Ata Akcil</td>
<td>S. D. University</td>
</tr>
<tr>
<td>Dr. Adhar Manna</td>
<td>The University of South Dakota</td>
</tr>
<tr>
<td>Dr. Cícero Flávio Soares Aragão</td>
<td>Federal University of Rio Grande do Norte</td>
</tr>
<tr>
<td>Dr. Gunnar Dahlen</td>
<td>Institute of odontology, Sahlgrenska Academy at University of Gothenburg</td>
</tr>
<tr>
<td>Dr. Pankaj Kumar Mishra</td>
<td>Vivekananda Institute of Hill Agriculture, (I.C.A.R.), ALMORA-263601, Uttarakhand</td>
</tr>
<tr>
<td>Dr. Benjamas W. Thanomsub</td>
<td>Srinakharinwirat University</td>
</tr>
<tr>
<td>Dr. Maria José Borrego</td>
<td>National Institute of Health – Department of Infectious Diseases</td>
</tr>
<tr>
<td>Name</td>
<td>Institution</td>
</tr>
<tr>
<td>-------------------------------</td>
<td>------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>Dr. Catherine Carrillo</td>
<td>Health Canada, Bureau of Microbial Hazards</td>
</tr>
<tr>
<td>Dr. Marcotty Tanguy</td>
<td>Institute of Tropical Medicine</td>
</tr>
<tr>
<td>Dr. Han-Bo Zhang</td>
<td>Laboratory of Conservation and Utilization for Bioresources</td>
</tr>
<tr>
<td>Dr. Ali Mohammed Somily</td>
<td>King Saud University</td>
</tr>
<tr>
<td>Dr. Nicole Wolter</td>
<td>National Institute for Communicable Diseases and University of the Witwatersrand, Johannesburg</td>
</tr>
<tr>
<td>Dr. Marco Antonio Nogueira</td>
<td>Universidade Estadual de Londrina</td>
</tr>
<tr>
<td>Dr. Bruno Pavoni</td>
<td>Department of Environmental Sciences University of Venice</td>
</tr>
<tr>
<td>Dr. Shih-Chieh Lee</td>
<td>Da-Yeh University</td>
</tr>
<tr>
<td>Dr. Satoru Shimizu</td>
<td>Horonobe Research Institute for the Subsurface Environment</td>
</tr>
<tr>
<td>Dr. Tang Ming</td>
<td>College of Forestry, Northwest A&amp;F University, Yangling</td>
</tr>
<tr>
<td>Dr. Olga Gortzi</td>
<td>Department of Food Technology, T.E.I. of Larissa</td>
</tr>
<tr>
<td>Dr. Mark Tarnopolsky</td>
<td>McMaster University</td>
</tr>
<tr>
<td>Dr. Sami A. Zabin</td>
<td>Al Baha University</td>
</tr>
<tr>
<td>Dr. Julia W. Pridgeon</td>
<td>Aquatic Animal Health Research Unit, USDA, ARS</td>
</tr>
<tr>
<td>Dr. Lim Yau Yan</td>
<td>Monash University Sunway Campus</td>
</tr>
<tr>
<td>Prof. Rosemeire C. L. R. Pietro</td>
<td>Faculdade de Ciências Farmacêuticas de Araraquara, Univ Estadual Paulista, UNESP</td>
</tr>
<tr>
<td>Dr. Nazime Mercan Dogan</td>
<td>PAU Faculty of Arts and Science, Denizli</td>
</tr>
<tr>
<td>Dr Ian Edwin Cock</td>
<td>Biomolecular and Physical Sciences</td>
</tr>
<tr>
<td>Prof. N K Dubey</td>
<td>Banaras Hindu University</td>
</tr>
<tr>
<td>Dr. S. Hemalatha</td>
<td>Department of Pharmaceutics, Institute of Technology, Banaras Hindu University, Varanasi. 221005</td>
</tr>
<tr>
<td>Dr. J. Santos Garcia A.</td>
<td>Universidad A. de Nuevo Leon</td>
</tr>
<tr>
<td>Name</td>
<td>Address</td>
</tr>
<tr>
<td>-----------------------------</td>
<td>-----------------------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>Dr. Somboon Tanasupawat</td>
<td>Department of Biochemistry and Microbiology, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok 10330 Thailand</td>
</tr>
<tr>
<td>Dr. Vivekananda Mandal</td>
<td>Post Graduate Department of Botany, Darjeeling Government College, Darjeeling – 734101. India</td>
</tr>
<tr>
<td>Dr. Shihua Wang</td>
<td>College of Life Sciences, Fujian Agriculture and Forestry University China</td>
</tr>
<tr>
<td>Dr. Victor Manuel Fernandes Galhano</td>
<td>CITAB-Centre for Research and Technology of Agro-Environment and Biological Sciences, Integrative Biology and Quality Research Group, University of Trás-os-Montes and Alto Douro, Apartado 1013, 5001-801 Vila Real, Portugal</td>
</tr>
<tr>
<td>Dr. Maria Cristina Maldonado</td>
<td>Instituto de Biotecnologia. Universidad Nacional de Tucuman, Argentina</td>
</tr>
<tr>
<td>Dr. Alex Soltermann</td>
<td>Institute for Surgical Pathology, University Hospital Zürich, Switzerland</td>
</tr>
<tr>
<td>Dr. Dagmara Sirova</td>
<td>Department of Ecosystem Biology, Faculty Of Science, University of South Bohemia, Branisovska 37, Ceske Budejovice, 37001, Czech Republic</td>
</tr>
<tr>
<td>Dr. E. O Igbinosa</td>
<td>Department of Microbiology, Ambrose Alli University, Ekpoma, Edo State, Nigeria.</td>
</tr>
<tr>
<td>Dr. Hodaka Suzuki</td>
<td>National Institute of Health Sciences, Japan</td>
</tr>
<tr>
<td>Dr. Mick Bosilevac</td>
<td>US Meat Animal Research Center, USA</td>
</tr>
<tr>
<td>Dr. Nora Lía Padola</td>
<td>Imunoquímica y Biotecnología- Fac Cs Vet-UNCPBA, Argentina</td>
</tr>
<tr>
<td>Dr. Maria Madalena Vieira-Pinto</td>
<td>Universidade de Trás-os-Montes e Alto Douro, Portugal</td>
</tr>
<tr>
<td>Dr. Stefano Morandi</td>
<td>CNR-Istituto di Scienze delle Produzioni Alimentari (ISPA), Sez. Milano, Italy</td>
</tr>
<tr>
<td>Dr Line Thorsen</td>
<td>Copenhagen University, Faculty of Life Sciences, Denmark</td>
</tr>
<tr>
<td>Dr. Ana Lucia Falavigna-Guilherme</td>
<td>Universidade Estadual de Maringá, Brazil</td>
</tr>
<tr>
<td>Dr. Baoqiang Liao</td>
<td>Dept. of Chem. Eng., Lakehead University, 955 Oliver Road, Thunder Bay, Ontario, Canada</td>
</tr>
<tr>
<td>Dr. Ouyang Jinping</td>
<td>Patho-Physiology department, Faculty of Medicine of Wuhan University, China</td>
</tr>
<tr>
<td>Dr. John Sorensen</td>
<td>University of Manitoba, Canada</td>
</tr>
<tr>
<td>Dr. Andrew Williams</td>
<td>University of Oxford, United Kingdom</td>
</tr>
<tr>
<td>Dr. Chi-Chiang Yang</td>
<td>Chung Shan Medical University, Taiwan, R.O.C.</td>
</tr>
<tr>
<td>Dr. Quanming Zou</td>
<td>Department of Clinical Microbiology and Immunology, College of Medical Laboratory, Third Military Medical University, China</td>
</tr>
<tr>
<td>Name</td>
<td>Institution and Location</td>
</tr>
<tr>
<td>-----------------------------</td>
<td>----------------------------------------------------------------</td>
</tr>
<tr>
<td>Prof. Ashok Kumar</td>
<td>School of Biotechnology, Banaras Hindu University, Varanasi, India</td>
</tr>
<tr>
<td>Dr. Chung-Ming Chen</td>
<td>Department of Pediatrics, Taipei Medical University Hospital, Taipei, Taiwan</td>
</tr>
<tr>
<td>Dr. Jennifer Furin</td>
<td>Harvard Medical School, USA</td>
</tr>
<tr>
<td>Dr. Julia W. Pridgeon</td>
<td>Aquatic Animal Health Research Unit, USDA, ARS, USA</td>
</tr>
<tr>
<td>Dr. Alireza Seidavi</td>
<td>Islamic Azad University, Rasht Branch, Iran</td>
</tr>
<tr>
<td>Dr. Thore Rohwerder</td>
<td>Helmholtz Centre for Environmental Research UFZ, Germany</td>
</tr>
<tr>
<td>Dr. Daniela Billi</td>
<td>University of Rome Tor Vergat, Italy</td>
</tr>
<tr>
<td>Dr. Ivana Karabegovic</td>
<td>Faculty of Technology, Leskovac, University of Nis, Serbia</td>
</tr>
<tr>
<td>Dr. Flaviana Andrade Faria</td>
<td>IBILCE/UNESP, Brazil</td>
</tr>
<tr>
<td>Prof. Margareth Linde Athayde</td>
<td>Federal University of Santa Maria, Brazil</td>
</tr>
<tr>
<td>Dr. Guadalupe Virginia Nevarez Moorillon</td>
<td>Universidad Autonoma de Chihuahua, Mexico</td>
</tr>
<tr>
<td>Dr. Tatiana de Sousa Fiuza</td>
<td>Federal University of Goias, Brazil</td>
</tr>
<tr>
<td>Dr. Indrani B. Das Sarma</td>
<td>Jhulelal Institute of Technology, Nagpur, India</td>
</tr>
<tr>
<td>Dr. Guanghua Wang</td>
<td>Northeast Institute of Geography and Agroecology, Chinese Academy of Sciences, China</td>
</tr>
<tr>
<td>Dr. Renata Vadkertiova</td>
<td>Institute of Chemistry, Slovak Academy of Science, Slovakia</td>
</tr>
<tr>
<td>Dr. Charles Hocart</td>
<td>The Australian National University, Australia</td>
</tr>
<tr>
<td>Dr. Guoqiang Zhu</td>
<td>University of Yangzhou College of Veterinary Medicine, China</td>
</tr>
<tr>
<td>Dr. Guilherme Augusto Marietto Gonçalves</td>
<td>São Paulo State University, Brazil</td>
</tr>
<tr>
<td>Dr. Mohammad Ali Faramarzi</td>
<td>Tehran University of Medical Sciences, Iran</td>
</tr>
<tr>
<td>Dr. Suppasil Maneerat</td>
<td>Department of Industrial Biotechnology, Faculty of Agro-Industry, Prince of Songkla University, Hat Yai, 90112, Thailand</td>
</tr>
<tr>
<td>Dr. Francisco Javier Las heras Vazquez</td>
<td>Almeria University, Spain</td>
</tr>
<tr>
<td>Dr. Cheng-Hsun Chiu</td>
<td>Chang Gung memorial Hospital, Chang Gung University, Taiwan</td>
</tr>
<tr>
<td>Dr. Ajay Singh</td>
<td>DDU Gorakhpur University, Gorakhpur-273009 (U.P.), India</td>
</tr>
<tr>
<td>Dr. Karabo Shale</td>
<td>Central University of Technology, Free State, South Africa</td>
</tr>
<tr>
<td>Dr. Lourdes Zélia Zanoni</td>
<td>Department of Pediatrics, School of Medicine, Federal University of Mato Grosso do Sul, Campo Grande, Mato Grosso do Sul, Brazil</td>
</tr>
<tr>
<td>Name</td>
<td>Institution</td>
</tr>
<tr>
<td>-------------------------------</td>
<td>-----------------------------------------------------------------------------</td>
</tr>
<tr>
<td>Dr. Tulin Askun</td>
<td>Balikesir University</td>
</tr>
<tr>
<td></td>
<td>Turkey</td>
</tr>
<tr>
<td>Dr. Marija Stankovic</td>
<td>Institute of Molecular Genetics and Genetic Engineering</td>
</tr>
<tr>
<td></td>
<td>Republic of Serbia</td>
</tr>
<tr>
<td>Dr. Scott Weese</td>
<td>University of Guelph</td>
</tr>
<tr>
<td></td>
<td>Dept of Pathobiology, Ontario Veterinary College, University of Guelph,</td>
</tr>
<tr>
<td></td>
<td>Guelph, Ontario, N1G2W1, Canada</td>
</tr>
<tr>
<td>Dr. Sabiha Essack</td>
<td>School of Health Sciences</td>
</tr>
<tr>
<td></td>
<td>South African Committee of Health Sciences, University of KwaZulu-Natal</td>
</tr>
<tr>
<td></td>
<td>Private Bag X54001, Durban 4000 South Africa</td>
</tr>
<tr>
<td>Dr. Hare Krishna</td>
<td>Central Institute for Arid Horticulture, Beechwal, Bikaner-334 006, Rajasthan</td>
</tr>
<tr>
<td></td>
<td>India</td>
</tr>
<tr>
<td>Dr. Anna Mensuali</td>
<td>Dept. of Life Science, Scuola Superiore, Sant’Anna</td>
</tr>
<tr>
<td>Dr. Ghada Sameh Hafez Hassan</td>
<td>Pharmaceutical Chemistry Department, Faculty of Pharmacy, Mansoura University</td>
</tr>
<tr>
<td></td>
<td>Egypt</td>
</tr>
<tr>
<td>Dr. Kátia Flávia Fernandes</td>
<td>Biochemistry and Molecular Biology, Universidade Federal de Goiás, Brasil</td>
</tr>
<tr>
<td>Dr. Abdel-Hady El-Gilany</td>
<td>Public Health &amp; Community Medicine, Faculty of Medicine, Mansoura University</td>
</tr>
<tr>
<td></td>
<td>Egypt</td>
</tr>
<tr>
<td>Dr. Hongxiong Guo</td>
<td>STD and HIV/AIDS Control and Prevention, Jiangsu provincial CDC, China</td>
</tr>
<tr>
<td>Dr. Konstantina Tsaousi</td>
<td>Life and Health Sciences, School of Biomedical Sciences, University of Ulster</td>
</tr>
<tr>
<td>Dr. Bhavnaben Gowan Gordhan</td>
<td>DST/NRF Centre of Excellence for Biomedical TB Research</td>
</tr>
<tr>
<td></td>
<td>University of the Witwatersrand and National Health Laboratory Service</td>
</tr>
<tr>
<td></td>
<td>P.O. Box 1038, Johannesburg 2000, South Africa</td>
</tr>
<tr>
<td>Dr. Ernest Kuchar</td>
<td>Pediatric Infectious Diseases, Wroclaw Medical University, Wroclaw Teaching</td>
</tr>
<tr>
<td></td>
<td>Hospital, Poland</td>
</tr>
<tr>
<td>Dr. Hongxiong Guo</td>
<td>STD and HIV/AIDS Control and Prevention, Jiangsu provincial CDC, China</td>
</tr>
<tr>
<td>Dr. Mar Rodriguez Jovita</td>
<td>Food Hygiene and Safety, Faculty of Veterinary Science, University of Extremadura, Spain</td>
</tr>
<tr>
<td>Dr. Jes Gitz Holler</td>
<td>Hospital Pharmacy, Aalesund. Central Norway Pharmaceutical Trust Professor Brochs gt. 6. 7030 Trondheim, Norway</td>
</tr>
<tr>
<td>Prof. Chengxiang FANG</td>
<td>College of Life Sciences, Wuhan University Wuhan 430072, P.R.China</td>
</tr>
<tr>
<td>Dr. Anchalee Tungtrongchitr</td>
<td>Siriraj Dust Mite Center for Services and Research Department of Parasitology,</td>
</tr>
<tr>
<td></td>
<td>Faculty of Medicine Siriraj Hospital, Mahidol University</td>
</tr>
<tr>
<td></td>
<td>2 Prannok Road, Bangkok Noi Bangkok, 10700, Thailand</td>
</tr>
</tbody>
</table>
Instructions for Author

Electronic submission of manuscripts is strongly encouraged, provided that the text, tables, and figures are included in a single Microsoft Word file (preferably in Arial font).

The cover letter should include the corresponding author’s full address and telephone/fax numbers and should be in an e-mail message sent to the Editor, with the file, whose name should begin with the first author’s surname, as an attachment.

Article Types
Three types of manuscripts may be submitted:

Regular articles: These should describe new and carefully confirmed findings, and experimental procedures should be given in sufficient detail for others to verify the work. The length of a full paper should be the minimum required to describe and interpret the work clearly.

Short Communications: A Short Communication is suitable for recording the results of complete small investigations or giving details of new models or hypotheses, innovative methods, techniques or apparatus. The style of main sections need not conform to that of full-length papers. Short communications are 2 to 4 printed pages (about 6 to 12 manuscript pages) in length.

Reviews: Submissions of reviews and perspectives covering topics of current interest are welcome and encouraged. Reviews should be concise and no longer than 4-6 printed pages (about 12 to 18 manuscript pages). Reviews are also peer-reviewed.

Review Process
All manuscripts are reviewed by an editor and members of the Editorial Board or qualified outside reviewers. Authors cannot nominate reviewers. Only reviewers randomly selected from our database with specialization in the subject area will be contacted to evaluate the manuscripts. The process will be blind review. Decisions will be made as rapidly as possible, and the Journal strives to return reviewers’ comments to authors as fast as possible. The editorial board will re-review manuscripts that are accepted pending revision. It is the goal of the AJMR to publish manuscripts within weeks after submission.

Regular articles
All portions of the manuscript must be typed double-spaced and all pages numbered starting from the title page.

The Title should be a brief phrase describing the contents of the paper. The Title Page should include the authors’ full names and affiliations, the name of the corresponding author along with phone, fax and E-mail information. Present addresses of authors should appear as a footnote.

The Abstract should be informative and completely self-explanatory, briefly present the topic, state the scope of the experiments, indicate significant data, and point out major findings and conclusions. The Abstract should be 100 to 200 words in length. Complete sentences, active verbs, and the third person should be used, and the abstract should be written in the past tense. Standard nomenclature should be used and abbreviations should be avoided. No literature should be cited.

Following the abstract, about 3 to 10 key words that will provide indexing references should be listed.

A list of non-standard Abbreviations should be added. In general, non-standard abbreviations should be used only when the full term is very long and used often. Each abbreviation should be spelled out and introduced in parentheses the first time it is used in the text. Only recommended SI units should be used. Authors should use the solidus presentation (mg/ml). Standard abbreviations (such as ATP and DNA) need not be defined.

The Introduction should provide a clear statement of the problem, the relevant literature on the subject, and the proposed approach or solution. It should be understandable to colleagues from a broad range of scientific disciplines.

Materials and methods should be complete enough to allow experiments to be reproduced. However, only truly new procedures should be described in detail; previously published procedures should be cited, and important modifications of published procedures should be mentioned briefly. Capitalize trade names and include the manufacturer’s name and address. Subheadings should be used. Methods in general use need not be described in detail.
Results should be presented with clarity and precision. The results should be written in the past tense when describing findings in the authors’ experiments. Previously published findings should be written in the present tense. Results should be explained, but largely without referring to the literature. Discussion, speculation and detailed interpretation of data should not be included in the Results but should be put into the Discussion section.

The Discussion should interpret the findings in view of the results obtained in this and in past studies on this topic. State the conclusions in a few sentences at the end of the paper. The Results and Discussion sections can include subheadings, and when appropriate, both sections can be combined.

The Acknowledgments of people, grants, funds, etc should be brief.

Tables should be kept to a minimum and be designed to be as simple as possible. Tables are to be typed double-spaced throughout, including headings and footnotes. Each table should be on a separate page, numbered consecutively in Arabic numerals and supplied with a heading and a legend. Tables should be self-explanatory without reference to the text. The details of the methods used in the experiments should preferably be described in the legend instead of in the text. The same data should not be presented in both table and graph form or repeated in the text.

Figure legends should be typed in numerical order on a separate sheet. Graphics should be prepared using applications capable of generating high resolution GIF, TIFF, JPEG or Powerpoint before pasting in the Microsoft Word manuscript file. Tables should be prepared in Microsoft Word. Use Arabic numerals to designate figures and upper case letters for their parts (Figure 1). Begin each legend with a title and include sufficient description so that the figure is understandable without reading the text of the manuscript. Information given in legends should not be repeated in the text.

References: In the text, a reference identified by means of an author’s name should be followed by the date of the reference in parentheses. When there are more than two authors, only the first author’s name should be mentioned, followed by ‘et al’. In the event that an author cited has had two or more works published during the same year, the reference, both in the text and in the reference list, should be identified by a lower case letter like ‘a’ and ‘b’ after the date to distinguish the works.

Examples:
Abayomi (2000), Agindotan et al. (2003), (Kelebeni, 1983), (Usman and Smith, 1992), (Chege, 1998; 1987a,b; Tijani, 1993, 1995), (Kumasi et al., 2001)

References should be listed at the end of the paper in alphabetical order. Articles in preparation or articles submitted for publication, unpublished observations, personal communications, etc. should not be included in the reference list but should only be mentioned in the article text (e.g., A. Kingori, University of Nairobi, Kenya, personal communication). Journal names are abbreviated according to Chemical Abstracts. Authors are fully responsible for the accuracy of the references.

Examples:


Short Communications

Short Communications are limited to a maximum of two figures and one table. They should present a complete study that is more limited in scope than is found in full-length papers. The items of manuscript preparation listed above apply to Short Communications with the following differences: (1) Abstracts are limited to 100 words; (2) instead of a separate Materials and Methods section, experimental procedures may be incorporated into Figure Legends and Table footnotes; (3) Results and Discussion should be combined into a single section.

Proofs and Reprints: Electronic proofs will be sent (e-mail attachment) to the corresponding author as a PDF file. Page proofs are considered to be the final version of the manuscript. With the exception of typographical or minor clerical errors, no changes will be made in the manuscript at the proof stage.
Fees and Charges: Authors are required to pay a $550 handling fee. Publication of an article in the African Journal of Microbiology Research is not contingent upon the author's ability to pay the charges. Neither is acceptance to pay the handling fee a guarantee that the paper will be accepted for publication. Authors may still request (in advance) that the editorial office waive some of the handling fee under special circumstances.

Copyright: © 2015, Academic Journals.
All rights Reserved. In accessing this journal, you agree that you will access the contents for your own personal use but not for any commercial use. Any use and or copies of this Journal in whole or in part must include the customary bibliographic citation, including author attribution, date and article title.

Submission of a manuscript implies: that the work described has not been published before (except in the form of an abstract or as part of a published lecture, or thesis) that it is not under consideration for publication elsewhere; that if and when the manuscript is accepted for publication, the authors agree to automatic transfer of the copyright to the publisher.

Disclaimer of Warranties

In no event shall Academic Journals be liable for any special, incidental, indirect, or consequential damages of any kind arising out of or in connection with the use of the articles or other material derived from the AJMR, whether or not advised of the possibility of damage, and on any theory of liability. This publication is provided "as is" without warranty of any kind, either expressed or implied, including, but not limited to, the implied warranties of merchantability, fitness for a particular purpose, or non-infringement. Descriptions of, or references to, products or publications does not imply endorsement of that product or publication. While every effort is made by Academic Journals to see that no inaccurate or misleading data, opinion or statements appear in this publication, they wish to make it clear that the data and opinions appearing in the articles and advertisements herein are the responsibility of the contributor or advertiser concerned. Academic Journals makes no warranty of any kind, either express or implied, regarding the quality, accuracy, availability, or validity of the data or information in this publication or of any other publication to which it may be linked.
African Journal of Microbiology Research

Table of Content: Volume 9 Number 31, 5 August, 2015

ARTICLES

Lactic acid bacteria associated with the digestive tract and skin of Sea bream (*Sparus aurata*) cultured in Tunisia
Ouissal Chahad Bourouni, Jorge Barros-Velázquez, Pilar Calo-Mata and Monia El Bour

Culturable bacterial diversity and hydrolytic enzymes from drass, a cold desert in India
Puja Gupta and Jyoti Vakhlu

Isolation and optimization of amylase producing bacteria and actinomycetes from soil samples of Maraki and Tewedros campus, University of Gondar, North West Ethiopia
Gebreselema Gebreyohannes

Action of proteases of the nematophagous fungi *Pochonia chlamydosporia* on *Ascaris suum* eggs of collared peccary (*Pecari tajacu*)
Filippe Elias de Freitas Soares, José Humberto de Queiroz, Jackson Victor de Araújo, Maria Gorete Ramos Rodrigues, Alexandre de Oliveira Tavela, Anderson Rocha Aguiar, Tracy Lacerda, Carolina Magri Ferraz, Maria Cristina Valdetaro Rangel, Thiago Senna, Andreia Luíza Araújo, Tarcízio de Paula Rego, Caio Colodette Sena and Fabio Ribeiro Braga
Lactic acid bacteria associated with the digestive tract and skin of Sea bream (Sparus aurata) cultured in Tunisia

Ouissal Chahad Bourouni¹, Jorge Barros-Velázquez², Pilar Calo-Mata² and Monia El Bour¹*

¹Institut National des Sciences et Technologies de la Mer (INSTM), Rue 2 Mars 1934, 2025 Salammbô; Tunis, Tunisia. ²Department of Analytical Chemistry, Nutrition and Food Science, LHICA, School of Veterinary Sciences, University of Santiago de Compostela, E-27002 Lugo, Spain.

Received 4 February, 2014; Accepted 27 July, 2015

Thirty-seven (37) enterococcal isolates were recovered from the skin and intestines of the sea bream (Sparus aurata), the most economically important fish species of the Mediterranean sea from Tunisian fish farming sites, to investigate their antimicrobial potential. All isolates were identified to the species level using genotypic tools. An investigation employing 16S rDNA sequencing in combination with randomly amplified polymorphic DNA-polymerase chain reaction (RAPD-PCR) highlighted the predominance of the Enterococcus faecium (46%) and E. faecalis (19%) species. Other species, such as E. sanguinicola (3 strains), E. casseliflavus (3 strains), E. gallinarum (2 strains), Carnobacterium sp. (1 strain), Aerococcus viridans (2 strains) and Vagococcus carniphilus (2 strains) were also identified. The susceptibility to different antibiotics in addition to the antibacterial activities were investigated for all species identified. The isolates were sensitive to vancomycin but were resistant to several antibiotics relevant for therapy in human and animal medicine. Antibacterial profiles assayed against 39 bacterial indicators (including food-borne and fish pathogenic bacteria in aquaculture as well as other spoilage bacteria) showed that 46% of the isolates exhibited a large inhibition spectrum mainly towards Listeria monocytogenes, Staphylococcus aureus, Aeromonas hydrophila, Aeromonas salmonicida, Vibrio anguillarum and Carnobacterium strains. Therefore, highly inhibitory enterococcal strains could potentially be used as probiotics in sea bream and other farming fish fields.

Key words: Enterococcus, lactic acid bacteria, aquaculture, probiotic, sea bream.

INTRODUCTION

The frequent usage of antimicrobial agents has led to the development of multiple antibiotic resistance (MAR) in bacteria and has reduced the efficacy of antibiotic treatment for human and animal diseases (Tendencia and de la Pena, 2001; Pandiyar et al., 2013). Several studies implicated the use of antimicrobials in the fish farming sector and its environment for the prevention and treatment of animal and plant infections as well as for
promoting growth (Serrano, 2005; Kümmerer, 2009; Martínez, 2009).

The culture practices for most farmed fish species are mostly semi-intensive or intensive and farms are often affected by widespread antibiotic resistance in pathogens (Aeromonas hydrophila, Aeromonas salmonicida, Edwardsiella tarda, Edwardsiella ictaluri, Vibrio anguillarum, Vibrio salmonicida, Pasteurella piscida and Yersinia ruckeri), which are currently treated with antibiotics (Ben Kahla-Nakbi et al., 2009). Thus, replacing drugs with effective and inexpensive probiotics is necessary to avoid resistance in fish farming sites and antibiotic residues in fish flesh destined for human consumption (Rengpipat et al., 2008).

Lactic acid bacteria (LAB) belong to the bacterial communities present in the normal intestinal flora of fish and exhibit probiotic properties for aquaculture applications. Previous studies on several fish farming applications have shown the antagonistic properties of LAB on fish pathogens (Gatesoupe 1991; Ringo et al., 1995; Gonzalez et al., 2000; Vijayabaskar and Somasundaran, 2008; Rengpipat et al., 2008).

Within the LAB group, Enterococcus spp. are widespread in the gastrointestinal tract of mammals, birds, reptiles, insects and are found in the intestinal contents of several healthy fish species and therefore they could be amended to animal food as probiotics to contribute to the health of farmed fish (Campos et al., 2006; Calo-Mata et al., 2007).

Gilt-head sea bream (Sparus aurata), which together with sea bass (Dicentrarchus labrax) represent the main fish species with high economic value cultured in Mediterranean aquaculture and the main marine fish farmed in Tunisia, is affected by infectious diseases and the abusive use of antibiotics (Zorrilla et al., 2003; Ben Kahla-Nakbi et al., 2007). Even though it is well known that intestinal microflora, especially LAB, might influence the growth and health of farmed fish, there is no information available to date about the composition of the intestinal microflora in the sea bream that are widely cultured in Tunisia. Thus, the present study was firstly designed to investigate the presence and type of LAB of both the skin and gastrointestinal tract of farmed sea bream and to inquire about their bioactive potential against bacterial pathogens. To do so, we have characterised a large collection of sea bream LAB by phenotypic and genotypic analysis (including 16S rRNA sequencing and RAPD-PCR) and carried out the screening of their antimicrobial susceptibility patterns and their ability to produce antibacterial compounds against spoilage and fish pathogenic bacteria.

**MATERIALS AND METHODS**

**Fish and experimental conditions**

Gilt-head sea bream (S. aurata) specimens were collected from a fish farm in Hergla (central coast of Tunisia). Fish specimens were sampled in a water-ice mixture and kept in ice for 3 h until they arrived at our laboratory. A total of 30 fish specimens with body weights of 180-220 g were examined. Skin patches (2x1 cm²) were aseptically excised and the intestinal content was removed by dissecting the fish, removing the intestines (to the pyloric caeca) and squeezing out the contents. The gut contents appeared as faecal matter. All samples were weighed and homogenised for 1 min in sterile plastic bags and a Stomacher (Seward, London, United Kingdom). Homogenates of skin or gut were serially diluted in 0.9% saline solution, and 0.1 ml volumes of appropriate dilutions were spread on the surface of MRS (de Man, Rogosa and Sharpe medium) and M17 plates (Oxoid, Ltd., London, UK). The plates were incubated aerobically for 48-72 h at 30°C, and the isolated colonies with typical characteristics, namely pure white and small (2-3 mm in diameter) with entire margins, were picked from each plate and transferred to MRS broth or M17 broth (Oxoid) for experimental use.

**Phenotypic characterisation of the bacterial strains**

Pure cultures of all the isolates were subjected to the standard tests: colony morphology, cell morphology, motility, Gram stain and the production of cytochrome oxidase and catalase, fermentation tests of glucose and lactose, H₂S and gas production, the ability to grow at 10°C and 45°C in media (Brain Heart Infusion) containing 6.5% NaCl at pH 9.6 (Schleifer and Klipper-Bälz, 1984). All the Gram-positive bacteria belonging to the LAB group were further tested by means of miniaturised API 50 CH biochemical tests (BioMérieux, Marcy L’Etoile, France). The results of the identification tests were interpreted using the APILAB PLUS software, version 4.0 (BioMérieux).

**Genetic identification of LAB strains**

DNA from the LAB was isolated from the pellets formed after spining 1 ml of overnight cultures in MRS broth at 7500 rpm for 10 min. Each pellic was re-suspended in 180 μl of lysis buffer (20 mM Tris-Ci pH8, 2 mM EDTA, 1.2% triton X-100, 20 mg/ml lysozyme). Each 10 ml of lysis buffer was prepared by mixing 4 ml of lysozyme (10 mg/ml, in bi-distilled water), 4 ml of 50 mM Tris-HCl, 200 μl of 100 mM EDTA, 120 μl of Triton X-100 and 1.68 ml of Milli-Q water. All the reagents were purchased from Sigma Chemical Co. (St. Louis, MO, USA). After an incubation step at 37°C for 2 h, 25 μl of proteinase K (10 mg/ml) (Sigma) was added, followed by incubation at 70°C for 30 min. Then, the bacterial DNA was purified from each extract by means of a DNeasy tissue minikit (QIAGEN Inc., Valencia, CA, USA), based on the use of micro-columns. The concentration of purified DNA extract was determined by measuring the fluorescence that developed using a Quanti-IT kit and a Qubit fluorimeter (Invitrogen).

The genetic characterisation of LAB isolates was performed by a PCR amplification of 16S rDNA using the universal set of primers pBFPL (forward: 5’-AGTTGTATCGTGCTCAACG-3’) and p806R (reverse: 5’-GGACTACCAGGGTATCTAAT-3’) that yield an 800 bp PCR product (McCabe et al., 1995). The amplification conditions were as follows: a previous denaturing step at 94°C for 7 min was coupled to 30 cycles of denaturation (94°C for 1 min), annealing (55°C for 1 min) and extension (72°C for 1 min) and to a final extension step at 72°C for 15 min. All the amplification assays comprised 100 ng of the template DNA, 25 μl of a master mix (BioMix, Bioline Ltd., London, UK), including the reaction buffer, dNTPs, magnesium chloride and Taq DNA polymerase, PCR water (Genaxis, Montigny le Bretonneux, France), and 5 μl of each oligonucleotide primer to achieve a final volume of 50 μl. All PCR assays were carried out on a BioCycler Thermal Cycler (BioRad Laboratories, Hercules, CA, USA). The PCR products were visualized in 2.5% horizontal agarose (MS-8, Pronadisa, Madrid,
E. faecium, E. A LADDERS D 5042, Sigma) was used as a

RESULTS

Isolation of microorganisms

From the different samples of sea bream, 37 microbial isolates were examined; 11 of these strains were isolated from the skin and 26 strains were from the intestinal content. The physiological and biochemical characteristics of the isolated LAB strains are shown in Table 2. Thus, all the isolates were Gram-positive, catalase-negative, non-motile, non-spor-forming and chain-forming cocci, able to ferment glucose and to grow at 10°C and 45°C and in media containing 6.5% NaCl. All the strains produced acid from glucose, fructose, arbutin, esculin, maltose and trehalose, but not from arabinose, inositol, starch, rhamnose, dulcitol, inuline, xylitol, turanose, lycose, fucose, arabin, ceto-glucanate, erythritol, xylose or adonitol.

Identification

The genomic DNA of all the isolates was purified and ca. 800-bp fragments of their 16S rDNA were amplified and sequenced. The alignment of the 16S rRNA sequences showed that all the strains exhibited very high homology (≥ 95%) among themselves and with other Enterococcus strains deposited in the GenBank database. The results of the alignments allowed the classification of nine intestinal strains as E. faecium, seven strains as E. faecalis, three strains as E. casseliflavus, one strain as Enterococcus gallinarum, two strains as E. sanguinicola, one strain as Carnobacterium sp., two strains as Aerococcus viridans and two other strains as Vagococcus carnipillus. However, ten enterococci could not be identified to the species level but could only be identified to the genus level (Table 2). From the skin, only four different species were isolated (E. faecium, E. faecalis, A. viridans and Carnobacterium sp.).

The dendrogram derived from the sequence homology comparison of 16S rRNA gene sequences of isolates with respect to the reference sequences from GenBank is

Antibacterial activity of LAB strains

The potential bacteriocin-producing strains were screened against a range of 39 indicator pathogenic and spoilage microorganisms (Table 1). The detection of bacteriocin activity in LAB strains was initially screened by means of a standardised agar disk diffusion method. Briefly, Muller-Hinton (Oxoid) agar plates were seeded with a bacterial lawn of each indicator strain at a 10⁵ CFU/ml concentration. Then, extracellular extracts were prepared by centrifugation, at 7,000 rpm for 15 min. of 48-h culture in MRS of each strain and the cell-free extract was sterilized by filtration through 0.22 µm (Millex GS, Millipore, St. Quentin, France). Twenty µl of each LAB strain extracellular extract were placed on 6-mm sterile disks (Oxoid) that had previously been placed on the agar plates. The plates were incubated overnight at 37°C, and the antimicrobial activity was detected by the appearance of translucent halos in the bacterial lawn surrounding the disks. A nisin-producing L. lactis strain was included as a positive control for the antimicrobial activity.

RAPD-PCR reaction

RAPD-PCR was performed using 200 ng of the template DNA and 25 µl of a master mix (BioMix, Bioline Ltd., London, UK), including the reaction buffer, dNTPs, magnesium chloride and Taq DNA polymerase and PCR water (Genaxis, Montigny le Bretonneux, France) and 14 pmol of M13 (S-GAGGTTGCCGGTTCT-3) (Andrighetto et al., 2008) to achieve a final volume of 50 µl. The amplification reactions were performed using a thermal cycler from Applied Biosystems (GeneAmp-PCR System 2700). The following reaction conditions were used: initial denaturation at 94°C for 5 min, followed by 13 cycles at 94°C for 60 s, annealing at 45°C for 60 s, extension at 72°C for 60 s, and final extension at 72°C for 15 min. Ten µl of the PCR products were separated and visualised using a 1.5% horizontal agarose gel (MS media, Spain) in a solution of 1XTAE buffer (Tris-acetate-EDTA) and ethidium bromide (10 mg/ml) with electrophoresis at 80 V. The 123-bp DNA ladder (DNA LADDERS D 5042, Sigma) was used as a size marker. To check the reproducibility, the PCR assays were performed at least three times each. In each reaction, a tube without the template DNA was included as a negative control.

Microbial sensitivity towards antibiotics

The bacterial sensitivity was determined by the agar diffusion method according to Chabbert (1982) using the following 16 antibiotics selected as representatives of the different classes of antimicrobial agents relevant for therapy in human and animal medicine: vancomycin (30 µg), penicillin G (10 µU), amoxicillin (25 µg), oxacillin (5 µg), cefoxitin (30 µg), ceftriaxone (30 µg), streptomycin (10 µg), tobramycin (10 µg), neomycin (30 µU), chloramphenicol (30 µg), tetracycline (30 µU), oleandomycin (15 µg), nitrofurantoin (300 µU), trimethoprim-sulphamidine (25 µg), rifampicin (30 µg) and oxolinic acid (30 µg). Five ml of overnight culture in MRS broth, of the LAB strains was spread out on the surface of Mueller-Hinton agar plates (Oxoid). Then, the paper disks that were impregnated with the antimicrobial agents were placed onto the agar plate. After overnight incubation at 20°C, the diameter of the zone of inhibition of bacterial growth around each disk was measured. Based on the zones of inhibition, a qualitative report of “susceptible”, “intermediate” or “resistant” was determined for the tested bacteria according to the French National Guidelines (Comité de l’Antibiogramme de la Société Française de Microbiologie, 1996).

Antibacterial activity of LAB strains

The potential bacteriocin-producing strains were screened against a

Prior to sequencing, the PCR products were purified by means of an ExoSAP-IT kit (GE Healthcare, Uppsala, Sweden). Direct sequencing was performed with a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). The same primers used for PCR were employed for the sequencing of both strands of the PCR products. The sequencing reactions were analysed in an automatic sequencing system (ABI 3730 XL DNA Analyser, Applied Biosystems) with the POP-7 system. Sequence homologies were searched using the BLAST tool (National Centre for Biotechnology Information). The alignment of the new sequences with other ones present in GenBank was accomplished using the ClustalX software (Larkin et al., 2007). Phylogenetic and molecular evolutionary analyses were conducted with the MEGA software (Kumar et al., 2008), using the neighbour-joining method (Saitou and Nei, 1987) and the Kimura 2-parameter with 1000 bootstrap replicates to construct distance-based trees.

RAPD-PCR was performed using 200 ng of the template DNA and 25 µl of a master mix (BioMix, Bioline Ltd., London, UK), including the reaction buffer, dNTPs, magnesium chloride and Taq DNA polymerase and PCR water (Genaxis, Montigny le Bretonneux, France) and 14 pmol of M13 (S-GAGGTTGCCGGTTCT-3) (Andrighetto et al., 2008) to achieve a final volume of 50 µl. The amplification reactions were performed using a thermal cycler from Applied Biosystems (GeneAmp-PCR System 2700). The following reaction conditions were used: initial denaturation at 94°C for 5 min, followed by 13 cycles at 94°C for 60 s, annealing at 45°C for 60 s, extension at 72°C for 60 s, and final extension at 72°C for 15 min. Ten µl of the PCR products were separated and visualised using a 1.5% horizontal agarose gels (MS-8, Pronadisa, Madrid, Spain) in a solution of 1XTAE buffer (Tris-acetate-EDTA) and ethidium bromide (10 mg/ml) with electrophoresis at 80 V. The 123-bp DNA ladder (DNA LADDERS D 5042, Sigma) was used as a size marker. To check the reproducibility, the PCR assays were performed at least three times each. In each reaction, a tube without the template DNA was included as a negative control.
Table 1. Pathogenic and spoilage indicator microorganisms used to test the antibacterial activities of LAB isolates.

<table>
<thead>
<tr>
<th>Code</th>
<th>Species</th>
<th>Origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>AmH01</td>
<td><em>Aeromonas hydrophila</em></td>
<td>ATCC 7966</td>
</tr>
<tr>
<td>BaC23</td>
<td><em>Bacillus cereus</em></td>
<td>ATCC 14893</td>
</tr>
<tr>
<td>BaP31</td>
<td><em>Bacillus pumilus</em></td>
<td>ATCC 7061</td>
</tr>
<tr>
<td>BaS05</td>
<td><em>Bacillus Subtilis ssp. Spizizenii</em></td>
<td>ATCC 6633</td>
</tr>
<tr>
<td>BxT01</td>
<td><em>Brochothrix thermosphacta</em></td>
<td>ATCC 11509</td>
</tr>
<tr>
<td>CbD21</td>
<td><em>Carnobacterium divergens</em></td>
<td>ATCC 35677</td>
</tr>
<tr>
<td>CbM01</td>
<td><em>Carnobacterium malvaromaticum</em></td>
<td>LHICA collection</td>
</tr>
<tr>
<td>EbA01</td>
<td><em>Enterobacter aerogenes</em></td>
<td>ATCC 13048</td>
</tr>
<tr>
<td>EbC11</td>
<td><em>Enterobacter cloacae</em></td>
<td>ATCC 13047</td>
</tr>
<tr>
<td>HaA02</td>
<td><em>Haemophilus alvei</em></td>
<td>ATCC 9760</td>
</tr>
<tr>
<td>KIox11</td>
<td><em>Klebsiella oxytoca</em></td>
<td>ATCC 13182</td>
</tr>
<tr>
<td>KIP02</td>
<td><em>Klebsiella planticola</em></td>
<td>ATCC 33531</td>
</tr>
<tr>
<td>KIPn21</td>
<td><em>Klebsiella Pneumoniae ssp. pneumoniae</em></td>
<td>ATCC 10031</td>
</tr>
<tr>
<td>Lb30A</td>
<td><em>Lactobacillus saerimneri</em></td>
<td>LHICA collection</td>
</tr>
<tr>
<td>MoM02</td>
<td><em>Morganella morganii ssp. morganii</em></td>
<td>ATCC 8076H</td>
</tr>
<tr>
<td>PhD11</td>
<td><em>Photobacterium damselae</em></td>
<td>ATCC 33539</td>
</tr>
<tr>
<td>PrM01</td>
<td><em>Proteus mirabilis</em></td>
<td>ATCC 14153</td>
</tr>
<tr>
<td>PrP11</td>
<td><em>Proteus penneri</em></td>
<td>ATCC 33519</td>
</tr>
<tr>
<td>PrV21</td>
<td><em>Proteus vulgaris</em></td>
<td>ATCC 9484</td>
</tr>
<tr>
<td>PsF12</td>
<td><em>Pseudomonas fluorescens</em></td>
<td>ATCC 13525</td>
</tr>
<tr>
<td>PsFr51</td>
<td><em>Pseudomonas fragi</em></td>
<td>ATCC 4973</td>
</tr>
<tr>
<td>PsG21</td>
<td><em>Pseudomonas gessardii</em></td>
<td>LHICA collection</td>
</tr>
<tr>
<td>SrM53</td>
<td><em>Serratia marcescens ssp. marcescens</em></td>
<td>ATCC 274</td>
</tr>
<tr>
<td>SyE21</td>
<td><em>Staphylococcus xylosus</em></td>
<td>ATCC 35983</td>
</tr>
<tr>
<td>SyX11</td>
<td><em>Stenotrophomonas maltophilia</em></td>
<td>ATCC 29971</td>
</tr>
<tr>
<td>SM03</td>
<td><em>Staphylococcus aureus</em></td>
<td>ATCC 13637</td>
</tr>
<tr>
<td>59</td>
<td><em>Staphylococcus aureus</em></td>
<td>ATCC 9144</td>
</tr>
<tr>
<td>4521</td>
<td><em>Lysteria monocytogenes</em></td>
<td>ATCC 35845</td>
</tr>
<tr>
<td>4032</td>
<td><em>Lysteria monocytogenes</em></td>
<td>NCTC 11994</td>
</tr>
<tr>
<td>1112</td>
<td><em>Lysteria monocytogenes</em> 1112</td>
<td>LHICA collection</td>
</tr>
<tr>
<td>CI34.1</td>
<td><em>Pseudomonas anguilliseptica</em></td>
<td>Seabream*</td>
</tr>
<tr>
<td>ACR5.1(AS)</td>
<td><em>Aeromonas salmonicida</em></td>
<td>Turbot*</td>
</tr>
<tr>
<td>CI52.1(VCI)</td>
<td><em>Vibrio anguillarum</em></td>
<td>Seabream*</td>
</tr>
<tr>
<td>ACC30.1</td>
<td><em>Photobacterium damselae ssp. piscida</em></td>
<td>Sole*</td>
</tr>
<tr>
<td>V62</td>
<td><em>Vibrio anguillarum</em></td>
<td>Seabream**</td>
</tr>
<tr>
<td>VF</td>
<td><em>Vibrio anguillarum</em></td>
<td>Seabass***</td>
</tr>
<tr>
<td>AF</td>
<td><em>Aeromonas salmonicida</em></td>
<td>Seabass***</td>
</tr>
<tr>
<td>V90.11.287(V287)</td>
<td><em>Vibrio anguillarum</em></td>
<td>Seabass****</td>
</tr>
<tr>
<td>AH2</td>
<td><em>Pseudomonas fluorescens</em></td>
<td><em>Lates niloticus</em>***</td>
</tr>
</tbody>
</table>

*Strains provided by Pr. J. L. Romalde (Spain). **Strain provided by Pr. G. Breuil (France). ***Strains provided by Pr. J. C. Raymond (France). ****Strains provided by Pr. L. Gram (Denmark).

shown in Figure 1. The dendrogram suggests a close relationship between our isolates and the reference strains. According to this classification, the ten isolates that were identified to the genus level could be assigned to *E. faecium* (8 strains), *E. gallinarum* (1 strain) and *E. sanguinicola* (1 strain).

**RAPD-PCR analysis**

Further genetic intra-specific characterisation of the isolates was performed by RAPD-PCR analysis with M13 primers. The dendrogram derived from the combination of amplification profiles obtained with primers M13 is
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>UPA10</td>
<td>Skin</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
<td>-</td>
<td></td>
<td></td>
<td></td>
<td>-</td>
<td>-</td>
<td></td>
<td>-</td>
<td>-</td>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>E. faecium</td>
</tr>
<tr>
<td>UPA45</td>
<td>Intestine</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td></td>
<td>-</td>
<td></td>
<td></td>
<td></td>
<td>-</td>
<td>-</td>
<td></td>
<td>-</td>
<td>-</td>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>E. faecium</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>UPA46</td>
<td>Intestine</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td></td>
<td>-</td>
<td></td>
<td></td>
<td></td>
<td>-</td>
<td>-</td>
<td></td>
<td>-</td>
<td>-</td>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>E. faecium</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>UPA49</td>
<td>Intestine</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
<td>-</td>
<td></td>
<td></td>
<td></td>
<td>+</td>
<td>+</td>
<td></td>
<td>+</td>
<td>+</td>
<td></td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>E. faecium</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>UPA51</td>
<td>Intestine</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
<td>-</td>
<td></td>
<td></td>
<td></td>
<td>-</td>
<td>+</td>
<td></td>
<td>-</td>
<td>-</td>
<td></td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>E. faecium</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>UPA60</td>
<td>Intestine</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
<td>-</td>
<td></td>
<td></td>
<td></td>
<td>-</td>
<td>+</td>
<td></td>
<td>+</td>
<td>+</td>
<td></td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>E. faecium</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>UPA61</td>
<td>Skin</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td></td>
<td>-</td>
<td></td>
<td></td>
<td></td>
<td>+</td>
<td>+</td>
<td></td>
<td>+</td>
<td>+</td>
<td></td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>E. faecium</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>UPA68</td>
<td>Intestine</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td></td>
<td>-</td>
<td></td>
<td></td>
<td></td>
<td>-</td>
<td>+</td>
<td></td>
<td>-</td>
<td>-</td>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>E. faecium</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>UPA71</td>
<td>Intestine</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td></td>
<td>-</td>
<td></td>
<td></td>
<td></td>
<td>-</td>
<td>+</td>
<td></td>
<td>-</td>
<td>-</td>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>E. faecium</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>UPA72</td>
<td>Intestine</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td></td>
<td>-</td>
<td></td>
<td></td>
<td></td>
<td>+</td>
<td>+</td>
<td></td>
<td>+</td>
<td>+</td>
<td></td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>E. faecium</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>UPA75</td>
<td>Intestine</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td></td>
<td>-</td>
<td></td>
<td></td>
<td></td>
<td>+</td>
<td>+</td>
<td></td>
<td>+</td>
<td>+</td>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>E. faecium</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>UPA77</td>
<td>Skin</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td></td>
<td>-</td>
<td></td>
<td></td>
<td></td>
<td>-</td>
<td>-</td>
<td></td>
<td>-</td>
<td>+</td>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>E. faecium</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>UPA82</td>
<td>Intestine</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
<td>-</td>
<td></td>
<td></td>
<td></td>
<td>-</td>
<td>-</td>
<td></td>
<td>-</td>
<td>-</td>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>E. faecium</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>UPA85</td>
<td>Intestine</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td></td>
<td>-</td>
<td></td>
<td></td>
<td></td>
<td>-</td>
<td>-</td>
<td></td>
<td>-</td>
<td>-</td>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>E. faecium</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>UPA86</td>
<td>Skin</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td></td>
<td>-</td>
<td></td>
<td></td>
<td></td>
<td>-</td>
<td>-</td>
<td></td>
<td>-</td>
<td>-</td>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>E. faecium</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>UPA87</td>
<td>Intestine</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td></td>
<td>-</td>
<td></td>
<td></td>
<td></td>
<td>-</td>
<td>-</td>
<td></td>
<td>-</td>
<td>-</td>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>E. faecium</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>UPA89</td>
<td>Intestine</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td>-</td>
<td>-</td>
<td></td>
<td>-</td>
<td>-</td>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>E. faecium</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>UPA100</td>
<td>Intestine</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td>-</td>
<td>-</td>
<td></td>
<td>-</td>
<td>-</td>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>E. faecium</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>UPA102</td>
<td>Skin</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td>-</td>
<td>-</td>
<td></td>
<td>-</td>
<td>-</td>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>E. faecium</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>UPA103</td>
<td>Intestine</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td>-</td>
<td>-</td>
<td></td>
<td>-</td>
<td>-</td>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>E. faecium</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>UPA104</td>
<td>Intestine</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td>-</td>
<td>-</td>
<td></td>
<td>-</td>
<td>-</td>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>E. faecium</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>UPA106</td>
<td>Intestine</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td>-</td>
<td>-</td>
<td></td>
<td>-</td>
<td>-</td>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>E. faecium</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>UPA110</td>
<td>Intestine</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td></td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td>-</td>
<td>-</td>
<td></td>
<td>-</td>
<td>-</td>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>E. faecium</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 2. Biochemical and phenotypical tests of the LAB isolates
Figure 1. Phylogenetic relationships according to the partial sequencing of the 16S rDNA gene of LAB isolates and reference strains from GenBank by means of the neighbor-joining method. (X) GenBank accession numbers of the LAB isolates.
shown as Figure 2. Thus, the RAPD analysis with M13 primers yielded a clear discrimination of the different Enterococcus species isolated, allowing their grouping into clusters corresponding to each species. At a similarity level of 40%, arbitrarily chosen for the defining species, four main clusters were observed. The Cluster 1 grouped isolates belonged to the species E. faecium. Cluster 2 could be divided into three subclusters, each of them grouping isolates belonging to E. casseliflavus, E. gallinarum and E. sanguinicola, respectively. The third

**Figure 2.** RAPD-PCR patterns of the isolates obtained by using the primers M13, and dendrogram obtained by UPGMA of correlation value of merged normalised RAPD-PCR patterns.
cluster also contained two subclusters that grouped isolates belonging to *A. viridans* and *V. carniphilus*. Finally, a fourth cluster grouped isolates belonging to *E. faecalis* species. The strain *Carnobacterium sp.* clustered as an independent strain. It is particularly interesting to note that the grouping of the isolates with the RAPD analysis was in agreement with the classification provided by 16S RNA sequencing.

After the comparison of the amplification profiles obtained for the isolates that were identified to the genus level with those generated for others strains, it was possible to assign 8 isolates to *E. faecium*, one isolate to *E. gallinarum* and one to *E. sanguinicola*, which confirmed the data provided by the dendrogram generated by 16S RNA sequencing. These results confirmed the data resulting from the dendrogram generated by the 16S RNA phylogenetic analysis.

**Microbial sensibility towards antibiotics**

All the strains tested were resistant to at least three of the antibiotics. Thus, resistance to oxacillin, cephalosporins (cefoxitin, ceftriaxon), aminoglicosids (tobramycin and neomycin), macrolides (oleandomycin) and oxolinic acid were common among the isolates (Figure 3). In contrast, penicillin, streptomycin, phenicol, tetracyclin, rifampicin, trimethoprim-sulphamid and nitrofurantoin were the most active antibiotics against the majority of the LAB isolates. Nevertheless, it is well known by now that the administration of nitrofurantoin is banned in fish and shellfish farming. Interestingly, all the strains were sensitive to vancomycin.

The resistance patterns of the enterococcal isolates indicated a considerable diversity of strain-specific antibiotypes. Thus, up to 18 different antibiotypes were characterised, including those with resistance to three to ten antimicrobial agents (Table 3). Five different resistance types against seven antibiotics, four resistance types against eight antibiotics, three resistance types against six and nine antibiotics and two resistance types against ten antibiotics were characterised (Table 3).

An analysis of the phenotypic relationships among the enterococci isolated from the skin and intestinal content of the fish was also carried out and showed that, among the 28 antimicrobial resistance patterns obtained, nine were specific to the isolates recovered from the intestines of the fish and that five patterns were specific of those recovered from the fish skin.

**Antibacterial activity by LAB isolates**

All the isolates were assayed for inhibitory production against 39 Gram-positive and Gram-negative indicator bacteria, including food-borne and fish pathogenic bacteria and other spoilage bacteria (Table1). Seventeen strains (46%) exhibited inhibitory activity against a large number of the indicator strains investigated (Figure 4). Greater inhibition was observed against *L. monocytogenes*, *S. aureus*, *A. hydrophila*, *A. salmonicida*, *V. anguillarum* and *Carnobacterium* strains (Table 4). The diameters of the inhibition halos were within the 6.5–20 mm range. Thus, we selected 12 strains that strongly inhibited a large number of indicators and generated inhibitory zones with diameters larger than 11 mm for future studies and to evaluate their potential use as probiotics.
Table 3. Antibiotypes of the LAB strains isolated from farmed sea bream.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Lab codes</th>
<th>No. of resistance</th>
<th>Type of antimicrobial agents</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enterococcus sp.</td>
<td>UPAA 60</td>
<td>10</td>
<td>N-RA-FOX-TE-OX-CRO-AX-OL-AR-TOB</td>
</tr>
<tr>
<td>Enterococcus faecium</td>
<td>UPAA 66</td>
<td></td>
<td>N-SXT-FOX-OX-CRO-AX-OL-AR-STR-TOB</td>
</tr>
<tr>
<td>Enterococcus faecium (6 strains)</td>
<td>UPAA 11/13/22/85/45/61</td>
<td>9</td>
<td>N-FOX-OX-CRO-AX-OL-AR-STR-TOB</td>
</tr>
<tr>
<td>Enterococcus faecalis</td>
<td>UPAA 21</td>
<td></td>
<td>N-FOX-OX-CRO-AX-OL-AR-STR-TOB</td>
</tr>
<tr>
<td>Enterococcus sanguinicola</td>
<td>UPAA 72</td>
<td></td>
<td>N-FOX-OX-CRO-AX-OL-AR-STR-TOB</td>
</tr>
<tr>
<td>Enterococcus faecalis</td>
<td>UPAA 26</td>
<td></td>
<td>N-FOX-TE-OX-CRO-AX-AR-STR-TOB</td>
</tr>
<tr>
<td>Enterococcus gallinarum</td>
<td>UPAA 82</td>
<td></td>
<td>N-FOX-TE-OX-CRO-AX-AR-STR-TOB</td>
</tr>
<tr>
<td>Enterococcus faecium (2 strains)</td>
<td>UPAA 89/34</td>
<td></td>
<td>N-FOX-TE-OX-CRO-AX-AR-STR-TOB</td>
</tr>
<tr>
<td>Enterococcus faecalis</td>
<td>UPAA 75</td>
<td>8</td>
<td>N-SXT-RA-FOX-OX-CRO-OL-AR-TOB</td>
</tr>
<tr>
<td>Enterococcus gallinarum</td>
<td>UPAA 106</td>
<td></td>
<td>N-FOX-OX-CRO-OL-AR-STR-TOB</td>
</tr>
<tr>
<td>Enterococcus faecalis (2 strains)</td>
<td>UPAA 102/38</td>
<td></td>
<td>N-FOX-OX-CRO-OL-AR-STR-TOB</td>
</tr>
<tr>
<td>Carnobacterium sp.</td>
<td>UPAA 77</td>
<td></td>
<td>N-FOX-OX-CRO-OL-AR-STR-TOB</td>
</tr>
<tr>
<td>Enterococcus faecalis</td>
<td>UPAA 100</td>
<td></td>
<td>FOX-OX-CRO-AX-OL-AR-STR-TOB</td>
</tr>
<tr>
<td>Enterococcus faecium</td>
<td>UPAA 5</td>
<td></td>
<td>N-SXT-RA-FOX-OX-OL-AR-TOB</td>
</tr>
<tr>
<td>Enterococcus faecalis</td>
<td>UPAA 52</td>
<td></td>
<td>N-FOX-OX-CRO-AX-OL-AR-TOB</td>
</tr>
<tr>
<td>Enterococcus faecim</td>
<td>UPAA 40</td>
<td>7</td>
<td>N-SXT-RA-FOX-OL-AR-TOB</td>
</tr>
<tr>
<td>Enterococcus faecim</td>
<td>UPAA 39</td>
<td></td>
<td>N-FOX-CRO-AX-AR-STR-TOB</td>
</tr>
<tr>
<td>Enterococcus faecim (2 strains)</td>
<td>UPAA 44/35</td>
<td></td>
<td>N-FOX-OX-CRO-OL-AR-TOB</td>
</tr>
<tr>
<td>Enterococcus casseliflavus</td>
<td>UPAA 104</td>
<td></td>
<td>N-FOX-OX-CRO-OL-AR-TOB</td>
</tr>
<tr>
<td>Vagococcus carnophilus (2 strains)</td>
<td>UPAA 46/51</td>
<td></td>
<td>N-FOX-OX-CRO-AR-STR-TOB</td>
</tr>
<tr>
<td>Enterococcus faecim</td>
<td>UPAA 110</td>
<td></td>
<td>N-FOX-OX-CRO-AR-STR-TOB</td>
</tr>
<tr>
<td>Enterococcus sanguinicola</td>
<td>UPAA 71</td>
<td></td>
<td>N-FOX-OX-CRO-AR-STR-TOB</td>
</tr>
<tr>
<td>Enterococcus sanguinicola</td>
<td>UPAA 49</td>
<td></td>
<td>FOX-OX-CRO-AX-AR-STR-TOB</td>
</tr>
<tr>
<td>Enterococcus casseliflavus</td>
<td>UPAA 103</td>
<td></td>
<td>N-C30-AX-OL-AR-STR</td>
</tr>
<tr>
<td>Aerococcus viridans</td>
<td>UPAA 86</td>
<td>6</td>
<td>F-C30-AX-OL-TOB</td>
</tr>
<tr>
<td>Enterococcus faecim</td>
<td>UPAA 87</td>
<td></td>
<td>N-FOX-OL-AR-STR-TOB</td>
</tr>
<tr>
<td>Enterococcus casseliflavus</td>
<td>UPAA 9</td>
<td></td>
<td>N-FOX-OX-CRO-AR-TOB</td>
</tr>
<tr>
<td>Aerococcus viridans</td>
<td>UPAA 68</td>
<td>3</td>
<td>OL-AR-TOB</td>
</tr>
</tbody>
</table>

AX, amoxicillin; OX, oxacillin; FOX, cefoxitin; CRO, ceftriaxon; STR, streptomycin; TOB, tobramycin; N, neomycin; C, chloramphenicol; TE, tetracyclin; OL, oleandomycin; FM, furans; SXT, trimethoprim-sulphamide; RA, rifampicin; AR, oxolinic acid.

**DISCUSSION**

The high mortality rates that occur in the larval phases of cultures of marine fish such as sea bream (*Sparus aurata*), which is one of the most valuable cultured species in Tunisia and other Mediterranean countries, cause great economic losses to aquaculture facilities in these countries. This mortality has been frequently attributed to bacterial infections (Toranzo et al., 1993; Villamil et al., 2003). Among the possible ways to prevent this problem whilst avoiding the extensive use of antibiotics, is the use of bacteria such as LAB (potential probiotics). This subject has received increasing attention during the last decade (Ringo and Gatesoupe, 1998; Gatesoupe, 1999; Vazquez et al., 2004; Anders et al., 2010). However, to our knowledge no study has described the isolation, screening and characterisation of lactic acid bacteria to be used as probiotics in sea bream or in other fish species from Tunisian aquaculture facilities. Therefore, this study was firstly designed to...
Table 4. Antimicrobial activity of the enterococcal isolates against Gram-positive and Gram-negative fish pathogenic and food spoilage microorganisms.

| Producers strain | Indicators strain | Amh01 | BaC23 | BaP31 | BaS05 | BaX01 | ClD01 | ClM01 | HaA02 | HaP21 | MeM02 | PmV21 | PsE51 | SmS04 | SyX11 | 59 | 4021 | 4032 | 1112 | AS | AF | V62 | VCI | V231 | V257 | VF |
|------------------|-------------------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-----|-----|-----|-----|----|-----|-----|-----|----|-----|-----|-----|-----|
| UPAA5            |                   | 7     | 8     | 8     | 10    | 10    | 6.5   | 7     | 6.5   | 6.5   | 9     | 9     | 8     | 10    | 9     | 10  | 8   |
| UPAA11           |                   | 7     | 7     |       | 10    | 10    | 9     | 8     | 16    | 13    | 10    | 16    | 11    | 10    | 10   | 11  | 10  | 11  | 10  | 8   |
| UPAA22           |                   | 8     | 7     |       | 9     | 10    |       | 9     | 10    | 10    | 10    | 9     |       |       | 9   | 9   |
| UPAA26           |                   | 10    | 7     |       | 9     | 10    |       |       |       |       |       | 10    |       |       |     |     |
| UPAA34           |                   | 8     | 7     | 10    | 6.5   | 17    | 10    |       |       |       |       | 16    | 18    | 10    | 11   | 11  | 10  | 12  | 11  |
| UPAA35           |                   | 9     | 7     | 10    | 6.5   | 18    | 12    |       |       |       |       | 16    | 16    | 11    | 12   | 11  | 12  | 11  |
| UPAA39           |                   | 8     | 7     | 10    | 6.5   | 17    | 12    |       |       |       |       | 16    | 16    | 11    | 13   | 10  | 12  |
|UPAA40           |                   | 8     |       | 6.5   | 14    | 10    |       |       |       |       |       | 18    | 18    | 9     | 8    | 15  | 11  | 11  | 8   |
|UPAA44           |                   | 20    | 11    |       |       |       |       |       |       |       | 18    | 14    | 10    | 10   | 14  | 10  |
|UPAA45           |                   |       |       |       |       |       |       |       |       |       | 10    | 12    | 10    | 10   | 10  | 10  |
| UPAA49           |                   | 9     | 7     | 8     | 6.5   |       |       |       |       |       | 10    | 10    | 9     | 10   | 9    |
| UPAA61           |                   | 7     | 9     | 8     | 6.5   |       |       |       |       |       | 11    | 11    | 10    | 10   | 10  | 11  | 10  |
| UPAA71           |                   | 10    | 7     | 10    | 6.5   |       |       |       |       |       | 11    | 11    | 10    | 10   | 10  | 11  | 10  |
| UPAA72           |                   | 10    | 7     |       | 9     | 9     |       |       |       |       | 10    | 10    | 9     | 10   | 9    |
| UPAA85           |                   | 7     | 8     |       |       |       |       | 7     | 9     | 9     | 10    | 9     | 12    | 12   | 8   |
| UPAA89           |                   | 7     | 9     |       |       |       |       | 7     | 10    | 9     | 10    | 10    | 10    | 10   |
| UPAA110          |                   | 7     | 8     | 6.5   | 9     | 6.5   | 7     |       |       |       | 13    | 10    | 11    | 10   | 12  |
| Results are expressed as diameters of the inhibition zone in mm. Indicator strains EbA01, EbC11, KLOX11, KLP02, Lb30A, PhD11, ACC30.1, CI34.1, PrM01, PsF12, PrP11, PsG21, SyE21, SmM03 were not inhibited by any LAB strain.

Isolate, identify and characterise LAB associated with the skin and intestines of healthy sea bass because these LAB isolated in situ are normal residents and are persistent in the skin and intestines of the hosts; therefore, the host immune system should tolerate them (Tannock, 1999). Remarkably, enterococci were found to be ubiquitous among the fish samples tested. Enterococci are part of the normal intestinal microbiota of humans and animals and are used as indicators of faecal contamination of recreational water, but they can also be isolated from natural environments that have not been contaminated by faecal material (Roberts et al., 2009). Their occurrence in fish and fish environments has been described before (Kanoe and Abe, 1988; Peterson and Dalsgaard, 2003; Michel et al., 2007). The identification of Enterococcus species by physiological tests has always been problematic because of their considerable phenotypic diversity (Park et al., 1999), and commercially available kits are frequently insufficient for an accurate identification (Angeletti et al., 2001). Hence, in this work phenotypic analyses were complemented with 16S rDNA phylogenetic analysis and RAPD cluster analysis. A high congruency between RAPD and phylogenetic clusters was observed in this work, which is in agreement with previous reports (Vancanneyt et al., 2002; Linaje et al., 2004). Our work identified E. faecium as the most commonly isolated Enterococcus species from European sea bream (Sparus aurata) (46% of
microbial isolates), followed by *E. faecalis* (19%), and, to a lesser extent *E. sanguinicola*, *E. casseliflavus* and *E. gallinarum*. Three species were also isolated: *Carnobacterium* sp. (one strain), two strains of *Aerococcus viridans* and two strains of *Vagococcus carnipilus*.

*E. faecalis*, *E. faecium* and other enterococcal species were not considered as indigenous flora of the fish gut (Ringo and Gatesoupe, 1998). However, Kanoe and Abe (1988) found high counts of *E. faecalis* and *E. faecium* in intestinal samples from marine fish, and Peterson and Dalsgaard (2003) noted the predominance of these two species among the enterococci isolated from integrated and traditional fish farms, suggesting that enterococci may be a member of the normal intestinal flora of fish. The high prevalence of *E. faecium* isolates recovered from our fish intestinal samples support this possibility. Identical results were found when we isolated LAB from a sea bass gut (*Dicentrarchus labrax*) (data not shown).

Concerning the genotypic characterisation of the isolates, and as reported in previous studies (Andrighetto et al., 2001; Suzzi et al., 2000; Vancanneyt et al., 2002), RAPD-PCR has been shown to be a valid and accurate method for the identification of enterococci and for detecting genetic diversity at strain level. The results obtained are in agreement with the phylogenetic analysis based on 16S rRNA sequences.

The antibiotic resistance trends among *Enterococcus* species have been extensively reviewed (Bonten et al., 2001; Franz et al., 2003). This matter has been mostly investigated for clinical and human enterococcal isolates because of their high clinical impact. In addition, a number of studies have attempted to compare the resistance spectra of different enterococci according to their human, animal or food origins (Ogier and Serror, 2008). The occurrence of antibiotic resistance among isolates seems to vary somewhat between studies and is often described to be strain- and region-dependent (Canzek et al., 2005) or may also differ according to the isolation method (Klein, 2003).

Enterococci are intrinsically resistant to low levels of penicillin, cephalosporins and aminoglycosides, and currently, these bacteria have acquired high-level resistance to vancomycin and/or aminoglycosides (Robert et al., 2009). The *Enterococcus* spp. isolated in our study were sensitive to vancomycin, penicillin and nitrofurantoin.

Remarkably, streptomycin, phenicol, tetracyclin, rifampicin and trimethoprim-sulphamid were the most active antibiotics against the majority of the bacterial isolates that were resistant to other antimicrobials tested (oxacillin, cephalosporins, aminoglycosids, macrolids and oxolinic acid). The frequent detection of antibiotic resistance among enterococci is probably due to the increasing use of antibiotics (Bhattacherjee et al., 1988; Pathak et al., 1993; Goni-Urriza et al., 2000; Rhodes et al., 2000), which is complicated by the efficient transfer mechanisms of resistance genes via conjugative plasmids and transposons operating in this bacterial group. Therefore, antibiotic resistance, at least to vancomycin, must be evaluated in these microorganisms before they can be used as probiotics and/or food additives. In our study, all the enterococcal strains tested were sensitive to vancomycin, which is a positive phenotype for selecting these strains as potential probiotics since vancomycin is one of the most clinically relevant antibiotics.

In our study, several bacteria inhibiting strains were selected from both the skin and intestines of the European sea bream (*S. aurata*). Other studies also showed that the skin and gastrointestinal tract of various fish species contain lactic acid bacteria that produce antibacterial compounds able to inhibit the growth of several microorganisms (Ringo 1999; Spanggaard et al., 2001; Rengpipat et al., 2008; Vijayabaskar and Somasundaram, 2008; Ringo, 2008). The antimicrobial spectra on inhibition observed for the *Enterococcus* species included several genera, which indicates a broad spectrum of activity against Gram-positive but also against Gram-negative pathogenic and spoilage organisms.

The fact that these LAB Gram-positive bacteria showed great inhibitory activity towards Gram-negative pathogens is interesting because it is in contrast to the belief that the inhibitory spectrum of LAB is generally restricted to other Gram-positive bacteria (Abee et al., 1995). In agreement with our results, some LAB have been reported to inhibit Gram-negative fish pathogens (Gildberg and Mikkelsen, 1998; Joborn et al., 1997; Ringo, 2008; Robertson et al., 2000). Also, a number of earlier studies have also shown that several marine bacteria produce inhibitory substances that inhibit bacterial pathogens in aquaculture systems (Nogami and Maeda, 1992; Austin et al., 1995; Rengpipat et al., 1998; Gram et al., 1999; Chahad et al., 2007).

Many strains of enterococci, mainly *E. faecalis* and *E. faecium*, are known to produce a variety of bacteriocins active against several pathogenic bacteria, such as *Listeria monocytogenes*, *Staphylococcus aureus*, and *Vibrio* sp. (Ogier and Serror, 2008). Given their commensal status, enterococci are used as probiotics for humans or farm animals (Tannock and Cook, 2002; Sayyed et al., 2014). The use of such bacteria to inhibit pathogens by the release of antimicrobial substances is now gaining importance in fish farming as a better and more effective alternative to the use of antibiotics to manage the health of these organisms (Vijayan et al., 2006; Iman et al., 2014).

This research has confirmed the abundance of enterococci in European sea bream, both at the skin and intestinal levels and proves that many of the enterococci exhibit inhibitory activity against a number of pathogen and spoilage strains. The selected enterococcal strains described in this study are currently under characterisation to elucidate their potential use as probiotic.
bacteria in aquaculture.

**Conflict of interests**

The authors did not declare any conflict of interest.

**ACKNOWLEDGMENTS**

This work was funded by the Spanish Agency for International Cooperation and Development (AECID, grant A/018689/08). We are grateful to Karola Boehme, Inmaculada Fdez. and Samuel Arlindo at USC for their excellent technical assistance. The authors are also grateful to Dr. Lone Gram from the Danish Institute of Piscicultural Research, to Dr. J. C. Raymond from the Comité National des Pêches maritimes et des Élevages Marins, France, to Dr. J. L. Romalde from USC, Spain and to Dr. G. Breuil from IFREMER Palavas, France, for the provision of microbial strains.

**REFERENCES**


Full Length Research Paper

Culturable bacterial diversity and hydrolytic enzymes from drass, a cold desert in India

Puja Gupta and Jyoti Vakhlu*

School of Biotechnology, University of Jammu, Jammu- 06, J & K, India.

Received 13 February, 2015; Accepted 27 April, 2015

Bacterial diversity of composite soil sample of drass was explored and screened for various hydrolytic enzymes. About 600 bacterial strains were isolated using six different growth media, that is, R2A, nutrient agar, King's B media, tryptic soy agar, Luria-Bertani agar and minimal media (100 isolates picked randomly from each media). These bacterial isolates were further differentiated on the basis of colony/cell morphology analysis, pigmentation and growth patterns. The 99 selected strains were subjected to amplified ribosomal DNA restriction analysis and the representative isolates from each cluster were chosen for 16S rRNA gene sequencing. Phylogenetic analysis led to the identification of 40 bacteria, grouped into three major phyla, Proteobacteria, Actinobacteria and Firmicutes differentiated into 17 different genera. These representatives were also investigated for hydrolases at low temperature (4-30°C). All the isolates secreted one or the other hydrolytic enzyme, that is, esterase (90%), lipase (80%), protease (32.5%), amylase (20%), cellulase (17.5%). These results indicate that culturable bacteria in soil of Drass could serve as an ideal candidate region for enzyme bioprospecting.

Key words: Pigment, drass, cultivable bacteria, phylogenetic diversity, enzyme production, soil.

INTRODUCTION

Microorganisms in the cold environments have received increasing attention during the past decade as they play a major role in food chains and biogeochemical cycles of these environments (Margesin and Miteva, 2011). Diverse bacteria have been recovered from polar environments such as Arctic and Antarctic. However, diversity in polar regions differ from several high-altitude regions such as the Himalayan ranges due to seasonal variations in temperature that results in different physical and biochemical properties. Studies on non-polar environments particularly Himalayan region have been largely carried out on glaciers and snow samples (Pradhan et al., 2010; Shivaji et al., 2011). There are very few reports on bacterial diversity of Himalayan hilly terrains. Microbes inhabiting these cold environments are extensively prospected for unique adaptabilities of their enzymes (de Pascale et al., 2008). Cold adapted enzymes have high catalytic efficiency and unique specificity at low and moderate temperatures, significantly at higher rate than the mesophilic counterparts (Gerday et al., 1997). These enzymes offer economic benefits through energy savings as they wipe out the requirement for expensive heating step. Due to their distinctive properties, these enzymes have also

*Corresponding author. E-mail: jyotivakhlu@gmail.com or jyotimetagenomic@gmail.com. Tel: 0941919-17624.

Author(s) agree that this article remain permanently open access under the terms of the Creative Commons Attribution License 4.0 International License
gained much attention regarding their potential for industrial and biotechnological applications, e.g. cold adapted proteases are well suited in waste management in cold environments where the degradation capabilities of endogenous micro-flora are reduced due to low temperatures (Pulicherla et al., 2011). In industries, for dehairing of hides and skins using psychrophilic proteases not only save energy but also reduce the impacts of toxic chemicals used in de-hairing (Joshi and Satyanarayana, 2013).

Amylases are one of the important industrial enzymes that have wide range of application such as food processing, fermentation and pharmaceutical industries. Cellulases are used in laundry detergents for exhibiting color brightness and removing the soil from cotton fibers, bio-polishing of fabric giving the finishing look of the product and producing stone washed look of denims (Aygan and Arikan, 2008; Sarvanan et al., 2013). Cellulases are gaining additional consideration in the enzyme market owing to their ability in the degradation of lignocellulosic biomass into biofuels and other products (Zhang et al., 2011). Lipases/esterases hold important position in the world enzyme market. The commercial use of lipases of cold origin is a billion dollar business. Psychrophilic lipases have attracted attention for synthesis of organic substances due to their inherent greater flexibility, whereas the activity of mesophilic and thermophilic enzymes are severely impaired by excess rigidity. These have great value for bioremediation and are widely used for degrading hydrocarbons present in contaminated soil (Aislabie et al., 2000; Paniker et al., 2006).

In the present study, an effort has been made to explore the bacterial diversity towards bioprospecting for hydrolytic enzymes from composite soil sample of Drass, located at 34.428152°N, 75.75118°E. It starts from the base of the Zojila pass (the Himalayan gateway to Ladakh), a trans-Himalayan region that separates the western Himalayan peaks from the Tibetan plateau (Figure 1). It is situated 60 km west of Kargil on the road to Srinagar with an average elevation of 3,280 m (10,764 ft) and experiences an altitude-influenced subarctic climate. The subarctic climate is characterized by long, usually very cold winters, and short, cool to mild summers. Winters start from mid-October and lasts in mid-May with temperature −22°C (−8°F) to as low as −45°C (−49°F) at the height of winter. Summers start in June and lasts till early September, with average temperatures near 15°C (59°F) and little precipitation. Annual precipitation is almost entirely concentrated in the months of December to May when Drass gets about 360 mm (14 inches) of snow.

**MATERIALS AND METHODS**

**Collection of soil sample and sampling site**

The soil samples (10) were collected from different regions of Drass mountains at 34.45°N, 75.77°E in North Himalayan range (J&K, Ladakh) during May 2010 and pooled into one composite sample. The soil was collected 1 cm deep into the earth by digging and collected in aseptic plastic bags (Shivaji et al., 2011). Hands, trowels were treated with 70% ethanol immediately before use. The samples were transported to the laboratory in ice and stored at...
Enumeration and isolation of heterotrophic bacteria

One gram of sample was aseptically weighed and homogenized in 9 ml sterile physiological water (0.86% NaCl) by vortexing vigorously. Six different media namely R2A, Nutrient agar, King B agar, Tryptic soy agar, Luria-Bertani agar Minimal media were used to isolate bacteria by plating $10^2$ and $10^3$ soil dilution with saline. All the growth media used in the present study were purchased from Himedia Pvt. Ltd India (Cat no. ≠ M962, M001, M1544, GM1151, M512, M290 respectively) and prepared according to the instructions given by the manufacture. The plates were incubated for 4-5 days in incubators at 4, 10, 20, 30°C temperature and the CFU/g of the soil was calculated.

Morphology and molecular identification

Preliminary taxonomic characteristics of the isolated bacteria were determined by colony morphology, pigment colour, growth pattern and biochemical analysis (Hamid et al., 2003). Pure cultures were cryopreserved in 50% glycerol at -80°C (New Brunswick, Efendorf). Genomic DNA was extracted by Hipura kit (Himedia, cat no. ≠ M512, M290 respectively) and prepared according to the instructions given by the manufacture. The plates were incubated for 4-5 days in incubators at 4, 10, 20, 30°C temperature and the CFU/g of the soil was calculated.

Screening for hydrolytic enzyme

Agar medium containing appropriate substrate and 1.5% agar (w/v) were inoculated with freshly grown cultures and incubated at 4, 10, 20, 30°C for 48 h. Different substrates for example 0.4% soluble starch (w/v), 0.4% (w/v) carboxymethylcellulose, 0.4% (w/v) tributyrin, olive oil (1%) and casein (0.4% w/v) were used for screening amylases, cellulases, esterases, lipases and proteases (Gangwar et al., 2009). For screening amylase and cellulase activity, incubated plates were developed by flooding the plates with iodine solution (1%) and washing with normal saline. For screening lipases, syringe filtrated olive oil (1%) and a florescent dye rhodamine B (0.001% w/v) was added to the autoclaved cooled growth medium with vigorous stirring. The plates containing bacterial cultures were observed for an orange fluorescence under UV light at 350 nm (Ranjitha et al., 2009).

RESULTS AND DISCUSSION

The present study is a first attempt to isolate and characterize the heterotrophic bacteria from soil of Drass, using different culturing conditions. Drass is the second coldest place in world after Siberia and its bacterial diversity (both cultivation dependent and independent) has not been unexplored so far. It is an established fact that cultivation based technique harvest only 1% of the bacteria and cultivation independent metagenomic techniques catalogue majority of the diversity. However, cultivation dependent conventional isolation techniques were employed as the aim of the present study was to isolate bacteria with hydrolytic activity that can be used commercially subsequently.

Bacterial isolation and characterization

Both oligotrophic and nutrient rich media were selected to obtain maximum cultivable bacteria. About 600 isolates were randomly selected (100 each from six different media: Nutrient agar, LB agar, King’s B agar, TSA, Minimal media, R2A agar) used in the study. Since the average summer and winter temperature varies between 4-30°C, the bacteria were isolated within this temperature range. The growth pattern of individual bacterial culture were studied and placed into psychrophilic (4-20°C), psychrotrophic (4-30°C), and psychrotolerant mesophilic (4-37°C), mesophilic (25-40°C) groups (Sahay et al., 2013) (Table 1). Maximum bacterial load (including pigmented and non-pigmented) 5.0±0.7x10^6 CFU/ml at 30°C CFU/ml was obtained using NA (Table 2) but maximum number of pigmented bacteria 2.9±0.17x10^6 were obtained with R2A media (Table 2). Pigment production was intense at 4°C and decreased with increase in incubation temperature which is in accordance with earlier studies on bacterial diversity of Puruogangri ice core (Zhang et al., 2008) and Himalayas (Venkatachalam et al., 2015). R2A is an oligotrophic medium and allows cultivation of many pigmented bacteria in particular that will not readily grow on fuller, complex organic media. R2A has been used to isolate bacteria from various cold environments e.g glaciers (Foght et al., 2004), marine surface waters (Agougue et al., 2005), ice cores (Zhang et al., 2008) and Antarctic soils (Dierser et al., 2010; Peeters et al., 2012). The pigments produced by these bacteria are reported to be carotenoids and has been co-related with cold adaptation of microorganisms by many workers (McDougal et al., 1998; Cho and Tiedje, 2000; Daniela et al., 2012; Mojib et al., 2013).

Diversity measures

Diversity indices were used to compare between the
Table 1. Taxonomic affiliations and phenotypic characterization of bacteria isolated from soils of Drass (J&K, Ladakh) determined by sequencing of 16S rRNA.

<table>
<thead>
<tr>
<th>Close representative Organisms/group</th>
<th>Phylum</th>
<th>Accession no.</th>
<th>Media used</th>
<th>Temperature range (°C)</th>
<th>Colony description</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Pseudomonas vranovensis</em> Dr1</td>
<td>Gammaproteobacteria</td>
<td>KF555604</td>
<td>King's B</td>
<td>Psychrotolerant mesophilic</td>
<td>Pale yellow</td>
</tr>
<tr>
<td><em>Pseudomonas putida</em> Dr2</td>
<td></td>
<td>JX978885</td>
<td>King's B</td>
<td>Psychrotolerant mesophilic</td>
<td>Pale yellow, transparent</td>
</tr>
<tr>
<td><em>Pseudomonas fuscovaginae</em> Dr5</td>
<td></td>
<td>JX978887</td>
<td>King's B</td>
<td>Psychrotolerant mesophilic</td>
<td>Pale yellow, transparent</td>
</tr>
<tr>
<td><em>Pseudomonas stutzeri</em> Dr12</td>
<td></td>
<td>KF555605</td>
<td>R2A</td>
<td>Psychrotolerant mesophilic</td>
<td>Yellow, wrinkled</td>
</tr>
<tr>
<td><em>Pseudomonas mandelli</em> Dr13</td>
<td></td>
<td>JN088486</td>
<td>Kings B</td>
<td>Psychrotrophic</td>
<td>Yellow, smooth</td>
</tr>
<tr>
<td><em>Pseudomonas psychrotolerans</em> Dr17</td>
<td></td>
<td>KF555606</td>
<td>R2A</td>
<td>Psychrotrophic</td>
<td>Yellow, wrinkled</td>
</tr>
<tr>
<td><em>Pseudomonas breneri</em> Dr29</td>
<td></td>
<td>KF555610</td>
<td>Kings B</td>
<td>Psychrotrophic</td>
<td>Yellow</td>
</tr>
<tr>
<td><em>Pseudomonas frederiksbergensis</em> Dr27</td>
<td>Gammaproteobacteria</td>
<td>KF555608</td>
<td>LB</td>
<td>Psychrotrophic</td>
<td>Pale yellowish</td>
</tr>
<tr>
<td><em>Acinetobacter calcoaceticus</em> Dr4</td>
<td></td>
<td>JX978886</td>
<td>LB</td>
<td>Psychrotolerant mesophilic</td>
<td>White, slimy</td>
</tr>
<tr>
<td><em>Acinetobacter radioresistens</em> Dr25</td>
<td></td>
<td>JX978884</td>
<td>NA</td>
<td>Psychrotolerant mesophilic</td>
<td>Cream</td>
</tr>
<tr>
<td><em>Serratia proteamaculans</em> Dr7</td>
<td></td>
<td>JX978888</td>
<td>LB</td>
<td>Psychrotolerant mesophilic</td>
<td>White, slimy</td>
</tr>
<tr>
<td><em>Pantoea agglomerans</em> Dr31</td>
<td></td>
<td>KF555611</td>
<td>R2A</td>
<td>Psychrotolerant mesophilic</td>
<td>Dark yellow</td>
</tr>
<tr>
<td><em>Pantoea agglomerans</em> Dr46</td>
<td></td>
<td>KM188063</td>
<td>R2A</td>
<td>Mesophilic</td>
<td>Yellow</td>
</tr>
<tr>
<td><em>Paracoccus marcusii</em> Dr32</td>
<td>Alphaproteobacteria</td>
<td>KF555612</td>
<td>R2A</td>
<td>Psychrotolerant mesophilic</td>
<td>orange</td>
</tr>
<tr>
<td><em>Bacillus safensis</em> Dr6</td>
<td></td>
<td>KF682429</td>
<td>LB</td>
<td>Mesophilic</td>
<td>White Opaque</td>
</tr>
<tr>
<td><em>Bacillus cereus</em> Dr8</td>
<td></td>
<td>JX978889</td>
<td>LB</td>
<td>Mesophilic</td>
<td>Cream slimy</td>
</tr>
<tr>
<td><em>Bacillus atrophaeus</em> Dr14</td>
<td></td>
<td>JX978892</td>
<td>NA</td>
<td>Mesophilic</td>
<td>Brownish black, rough</td>
</tr>
<tr>
<td><em>Bacillus simplex</em> Dr18</td>
<td></td>
<td>JN088488</td>
<td>NA</td>
<td>Psychrotolerant mesophilic</td>
<td>Cream slimy</td>
</tr>
<tr>
<td><em>Bacillus spp.</em> Dr22</td>
<td></td>
<td>JN088491</td>
<td>NA</td>
<td>Psychrotrophic</td>
<td>Cream</td>
</tr>
<tr>
<td><em>Bacillus vallismortis</em> Dr38</td>
<td></td>
<td>KF555618</td>
<td>LB</td>
<td>Mesophilic</td>
<td>Black</td>
</tr>
<tr>
<td><em>Bacillus thuringiensis</em> Dr45</td>
<td>Firmicutes</td>
<td>KF555624</td>
<td>NA</td>
<td>Psychrotrophic</td>
<td>Off-white</td>
</tr>
<tr>
<td><em>Staphylococcus equorum</em> Dr34</td>
<td></td>
<td>KF555614</td>
<td>LB</td>
<td>Psychrotolerant mesophilic</td>
<td>White</td>
</tr>
<tr>
<td><em>Sporosarcina psychrophila</em> Dr35</td>
<td></td>
<td>KF555615</td>
<td>MM</td>
<td>Psychrotolerant</td>
<td>Beige, shiny</td>
</tr>
<tr>
<td>*Sporosarcina psychrophila Dr41</td>
<td></td>
<td>KF555620</td>
<td>R2A</td>
<td>Psychrotrophic</td>
<td>Brownish</td>
</tr>
<tr>
<td><em>Exiguobacterium sibiricum</em> Dr19</td>
<td></td>
<td>JX978893</td>
<td>MM</td>
<td>Psychrotrophic</td>
<td>Orange, smooth</td>
</tr>
<tr>
<td><em>Exiguobacterium undae</em> Dr28</td>
<td></td>
<td>KF555609</td>
<td>R2A</td>
<td>Psychrotrophic</td>
<td>Light orange</td>
</tr>
<tr>
<td><em>Planomicrobium koreense</em> Dr24</td>
<td></td>
<td>JX978895</td>
<td>TSA</td>
<td>Psychrotolerant mesophilic</td>
<td>Orange</td>
</tr>
<tr>
<td><em>Arthrobacter agilis</em> Dr16</td>
<td></td>
<td>JX978896</td>
<td>R2A</td>
<td>Psychrotrophic</td>
<td>Rose red, smooth</td>
</tr>
<tr>
<td><em>Arthrobacter crystalloprietes</em> Dr37</td>
<td></td>
<td>KF555617</td>
<td>R2A</td>
<td>Psychrotrophic</td>
<td>Light yellow</td>
</tr>
<tr>
<td><em>Mycetocella reblochoni</em> Dr23</td>
<td>Actinobacteria</td>
<td>HE774268.1</td>
<td>R2A</td>
<td>Psychrotrophic</td>
<td>Yellow, smooth</td>
</tr>
<tr>
<td><em>Mycetocella reblochoni</em> (Dr42)</td>
<td></td>
<td>KF555621</td>
<td>TSA</td>
<td>Psychrotolerant mesophilic</td>
<td>Light yellow</td>
</tr>
<tr>
<td><em>Kocuria Polaris</em> (Dr20)</td>
<td></td>
<td>KF682428</td>
<td>R2A</td>
<td>Psychrotrophic</td>
<td>Red and smooth</td>
</tr>
<tr>
<td><em>Kocuria rosea</em> (Dr33)</td>
<td></td>
<td>KF555613</td>
<td>R2A</td>
<td>Psychrotrophic</td>
<td>Dark pink</td>
</tr>
</tbody>
</table>
Table 1. Contd.

<table>
<thead>
<tr>
<th>Bacterial isolates</th>
<th>Accession numbers</th>
<th>Media</th>
<th>Growth type</th>
<th>Colony/cell morphology</th>
<th>Pigmentation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rhodococcus erythropolis (Dr10)</td>
<td>JX978890</td>
<td>MM</td>
<td>Psychrotrophic</td>
<td>Pale yellow, slimy</td>
<td></td>
</tr>
<tr>
<td>Rhodococcus gingshengi (Dr21)</td>
<td>JX978894</td>
<td>R2A</td>
<td>Psychrotolerant mesophilic</td>
<td>Light pink</td>
<td></td>
</tr>
<tr>
<td>Rhodococcus erythropolis (Dr36)</td>
<td>KF555616</td>
<td>MM</td>
<td>Psychrotrophic</td>
<td>White, very slimy</td>
<td></td>
</tr>
<tr>
<td>Citricoccus alkalitolerans (Dr40)</td>
<td>KF555619</td>
<td>R2A</td>
<td>Psychrotolerant mesophilic</td>
<td>Light yellow</td>
<td></td>
</tr>
<tr>
<td>Dietzia schimae (Dr43)</td>
<td>KF555622</td>
<td>TSA</td>
<td>Psychrotolerant mesophilic</td>
<td>reddish orange</td>
<td></td>
</tr>
<tr>
<td>Micrococcus luteus (Dr44)</td>
<td>KF555623</td>
<td>NA</td>
<td>Mesophilic</td>
<td>Yellow</td>
<td></td>
</tr>
</tbody>
</table>

Table 2. Bacteria load (pigmented and non-pigmented) on six different media at different temperatures.

<table>
<thead>
<tr>
<th>Growth media</th>
<th>C.F.U at 4°C (P)</th>
<th>C.F.U at 10°C (P)</th>
<th>C.F.U at 20°C (P)</th>
<th>C.F.U at 30°C (P)</th>
</tr>
</thead>
<tbody>
<tr>
<td>R2A</td>
<td>0.5±0.15 x10⁶</td>
<td>0.2±0.25 x10⁶</td>
<td>1.2±0.05 x10⁶</td>
<td>2±0.09 x10⁶</td>
</tr>
<tr>
<td>NA</td>
<td>0.07±0.02 x10⁶</td>
<td>0.13±0.02 x10⁶</td>
<td>0.1±0.01 x10⁶</td>
<td>0.1±0.01 x10⁶</td>
</tr>
<tr>
<td>LB</td>
<td>0.18±10 x10⁶</td>
<td>0.22±0.05x10⁶</td>
<td>0.4±0.02 x10⁶</td>
<td>1.6±0.06 x10⁶</td>
</tr>
<tr>
<td>Kings B</td>
<td>0.3±10 x10⁶</td>
<td>0.1±0.07 x10⁶</td>
<td>0.4±0.16 x10⁶</td>
<td>0.6±0.3 x10⁶</td>
</tr>
<tr>
<td>TSA</td>
<td>0.2±0.10 x10⁶</td>
<td>0.3±0.12x10⁶</td>
<td>0.3±0.02 x10⁶</td>
<td>0.6±0.03 x10⁶</td>
</tr>
<tr>
<td>MM</td>
<td>0.2±0.09 x10⁶</td>
<td>0.1±0.05 x10⁶</td>
<td>0.4±0.15 x10⁶</td>
<td>0.6±0.3 x10⁶</td>
</tr>
</tbody>
</table>

C.F.U counted as cells/ml; Experiments were conducted in triplicates and the data are expressed as mean± SD.

communities obtained by using different media. More community complexity was found using R2A media (Figure 2 and Table 3). Overall Shannon-Wiener index (H) was 3.2, that is in accordance with previous reports from Himalayan bacterial diversity (Pradhan et al., 2010; Shivaji et al., 2011, Yadav et al., 2014).

Phylogenetic analysis of 16S rDNA sequences of isolates

Bacterial isolates were screened for duplicity by colony/cell morphology analysis, pigmentation, conventional biochemical tests that narrowed the 600 isolates into 99 isolates. These selected isolates were subjected to 16S rRNA gene amplification followed by restriction digestion with Alu I and Hha I. On the basis of ARDRA profiling, representative isolate from each cluster were sequenced and the nucleotide sequences were deposited in the NCBI GenBank database (Accession numbers: JX978884-JX978891, JX978892-JX978896, JN088486, JN088488, JN088491, KF555604-KF555606, KF555608-KF555624, KF682428, KF682429 and HE774268, KM188063). The nearest phylogenetic neighbor of all the 40 representative isolates were identified in the National Centre for Biotechnology Information (NCBI) (Table 1).

Drass isolates represented both Gram-positive and negative heterotrophic bacteria belonging to three major phylogenetic groups organized into three clusters, Proteobacteria (37.5%), Firmicutes (32.5%) and Actinobacteria (30%) (Figure 3). Proteobacteria dominates (37.5%) the culturable bacterial diversity of Drass with Gammaproteobacteria (35%) as the dominant class represented by genera Pseudomonas, Acinetobacter, Serratia and Pantoea. Pseudomonads represented the dominant genera among Gammaproteobacterium. Alphaproteobacteria is however represented by single genera, that is, Paracoccus (Dr32) (Figure 3). The results are in accordance with the previous studies on Himalayan that reports Firmicutes, Actinobacteria and Proteobacteria as the most common phylum (Shivaji et al., 2011).

Bacterial isolates showed 99% similarity with the reference sequences in the Genbank except for Dr 46 that showed 96% similarity with Pantoea agglomerans (Figure 4). DNA-DNA hybridization
Figure 2. Diversity of pigmented and non-pigmented bacteria on six different media. NA, Nutrient agar; LB, Luria-Bertani agar; MM, minimal media; TSA, tryptic soy agar.

Table 3. OTUs and Shannon-Wiener index represented by bacteria on different growth media.

<table>
<thead>
<tr>
<th>Growth media</th>
<th>OTUs</th>
<th>Shannon-Wiener index</th>
</tr>
</thead>
<tbody>
<tr>
<td>R2A</td>
<td>14</td>
<td>2.54</td>
</tr>
<tr>
<td>LB</td>
<td>8</td>
<td>2.07</td>
</tr>
<tr>
<td>NA</td>
<td>6</td>
<td>1.79</td>
</tr>
<tr>
<td>Kings B</td>
<td>5</td>
<td>1.60</td>
</tr>
<tr>
<td>Minimal media</td>
<td>4</td>
<td>1.38</td>
</tr>
<tr>
<td>TSA</td>
<td>3</td>
<td>1.09</td>
</tr>
</tbody>
</table>

Exiguobacterium are adapted to long-term freezing at temperatures as low as -12°C where intracellular water is not frozen and grow at subzero temperatures, displaying several feature of psychrophiles, such as membranes composition. Genus Pantoea (Selvakumar et al., 2008; Venkatachalam et al., 2015), Dietzia (Mayilraj et al., 2006), Staphylococcus and Citricoccus (Yadav et al., 2015) have been reported from Indian Himalayas. Members of the genus Paracoccus have been reported from Qinghai-Tibet Plateau permafrost (Zhu et al., 2013).

Extracellular hydrolytic enzyme activity

Cold-active enzymes from microbial sources have potential applications in biotechnology, agriculture and medicine (Feller, 2007; Tropeano et al., 2012; Moreno et al., 2013). The representative isolates were screened for their extracellular hydrolytic enzyme activity viz., esterase, lipases, protease, amylase and cellulose.
Interestingly, 90% of the isolates were esterase producers and out of them 80% were lipase producers, 32.5% were protease producers, 20% were amylase producers and 17.5% are cellulase producers. The comparative profile of the hydrolytic enzymes produced by bacterial isolates of Drass has been represented (Figure 5). *Arthrobacter agilis* Dr16 and *Kocuria Polaris* Dr20 were the best esterase producers and the enzymes produced were active at low temperatures (10°C). *Mycetocola reiblochoni* Dr23 and *Planomicrobium koreense* Dr24 though best protease producers, do not degrade skimmed milk below 20°C. *Bacillus cereus* Dr8 and *Acinetobacter radioresistens* Dr25 were multiple hydrolases producer at low temperatures (10°C). The results clearly indicated that these enzymes can be characterized for exploitation at industrial level. Gangwar and coworkers 2009 worked on the bacterial diversity isolated from soil samples from the western Himalayas, India and reported that 62% of the bacterial isolates produced lipase followed by protease, 54%, Amylase 28% and only 11% have cellulase activity (Gangwar et al., 2009). *Pseudomonas*, *Bacillus*, *Arthrobacter*, *Exiguobacterium*, *Mycetocola*, *Pantoea*, *Acinetobacter* and *Serratia* have been identified as hydrolytic enzyme producer in previous study on Himalayas (Salwan et al., 2010; VenKatachalam et al., 2015). Although, in addition other genera especially *Kocuria*, *Rhodococcus* and *Planomicrobium* isolated in our study also showed hydrolytic enzyme activity.
Figure 4. Neighbour joining tree based 16S rRNA sequences showing the positions of strain Dr46 *Pantoea* sp. and representatives of some other taxa. Bar, 0.01 substitutions per nucleotide position. *Planomicrobium Koreense* BPLa14 was used as an outgroup.
Conclusion

Though earth is dominated by cold habitats but most of the diversity studies and bioprospecting has been done either on thermophilic/thermotolerent or mesophillic bacteria. Recently, focus on exploring cold environments for diversity and bioprospecting has increased due to energy concerns and ecological reasons. Drass, located in the Western Himalayas, the second coldest place in India was found to be a rich source of novel bacteria and their produce. Interestingly Dr46 has low percentage similarity with the reference strain *Pantoea* and could probably be new species. The low percentage of probable novel bacteria isolated despite using six media and starting from 600 isolates suggests that more media formulation need to be tried and larger population needs to be characterized. Further screening for hydrolytic enzyme activity resulted in screening of some of the...
multiple hydrolase producers for industrial use and not merely cataloguing them as is possible in the cultivation independent approach.

Conflict of interest

The authors declare that there is no conflict of interest.

ACKNOWLEDGEMENTS

This study was supported by DBT (BT/PR11727/BCE/08/720/2008) and CSIR (9/100/0177)K13-EMR-I, Government of India.

REFERENCES


Full Length Research Paper

Isolation and optimization of amylase producing bacteria and actinomycetes from soil samples of Maraki and Tewedros campus, University of Gondar, North West Ethiopia

Gebreselema Gebreyohannes

Department of Biology, College of Natural and Computational Sciences, University of Gondar, P. O. Box 196, Gondar, Ethiopia.

Received 21 July, 2014; Accepted 15 June, 2015

The objective of the present study was to isolate, identify and optimize potential amylase producing bacteria and actinomycetes from soil samples. The soil samples were collected from Maraki and Tewedros campus, University of Gondar. Isolation was done by serial dilution and spread plate method. Primary screening of amylolytic activity of the isolates was performed by starch agar plate method. The submerged state fermentation method followed for the production of amylase by the optimization of temperature, pH, fermentation time and substrate concentration. From the soil samples, 18 isolates were identified and subjected to primary screening for amylolytic activity. Of which, five isolates were observed with maximum amylolytic activity during the primary screening. During the submerged state fermentation, maximum amylase activity was observed at 48 h and then declined. The optimum temperature observed for maximum amylase activity of *Bacillus* was 40°C and *Streptomyces* at 37°C. The highest amylase activity was observed at neutral pH and 4% of starch concentration. The colony morphology, Gram reaction, biochemical tests and Bergey’s manual of determinative bacteriology confirm the promising isolates belong to the genus *Bacillus* and *Streptomyces*. This preliminary study could provide base line information for the discovery of novel microbes from the natural resources for the production of amylase which will be used for multipurpose.

Key words: Amylase, isolation, optimization, submerged state fermentation.

INTRODUCTION

Amylase is an enzyme obtained from the microbes has been used by many industries as a source for production of foods and beverages. With the utilization of microorganisms it is possible to produce large scale and also easily manipulated for desired products (Sumrin et al., 2011). In general, enzymes produced from fungal and bacterial sources have many applications in industries (Aiyer, 2005). In addition, recent advancement in
biotechnological tool, utilization of amylase has widened in clinical research, medical chemistry and starch analytical chemistry. Earlier literatures highlighted that bacterial strains from the genus *Bacillus*, *Pseudomonas* and *Clostridium* and from the genus *Streptomyces* have been used to synthesize amylase (Kafilzadeh et al., 2012; Oyeleke et al., 2010). Multi-potential application and demand pave the way for increasing indigenous amylase production and searching for more efficient processes (Hmidet et al., 2009). In Ethiopia, there is no much work done in this area of research. The country has several undisturbed natural soil habitat. In this research work soil samples were collected from various locations of Maraki and Tewedros campus since these areas are abundant in plant biodiversity and soil types. Therefore, to contribute new knowledge in scientific world amylase producing microorganisms were isolated, identified, optimized and reported for the first time from this study area.

MATERIALS AND METHODS

Sample collection

Soil sample were collected from five locations in Maraki and Tewedros campus, University of Gondar, Ethiopia. The study area is located in the latitude and longitude of 12° 35' 21" N / 37° 26' 39" E. From the selected area, 100 g of top soil samples was collected after careful removal of debris in the collection site. The soil samples were collected by using a sterile spatula, kept in the polyethylene bag and transported to the microbiology laboratory for further analysis. Stock soil samples were stored at 4°C in a refrigerator for subsequent analysis.

Screening of potential amylase producing bacteria and actinomycetes by using starch hydrolysis test

Ten gram of soil sample was suspended in 90 mL of sterile saline water in a conical flask and mixed by vortex mixer. From this 10 mL of the diluted suspension was transferred into three conical flasks containing 90 mL of sterile saline water serially. From each conical flasks, 0.1 mL was transferred into starch agar plates (meat extract 3 g/L; peptic digest of animal tissue 5 g/L; soluble starch 2 g/L; agar 15 g/L; pH 7.2 ± 0.1) in triplicate. Then, the sample was distributed evenly by using L-shaped glass rod and incubated at 37°C for 24 h. After incubation period, colonies were further sub-cultured on the respective medium to obtain pure isolates and maintained at 4°C in a refrigerator for further investigation. The isolates were screened for amylolytic activity by streaking on the starch agar plates and incubated at 37°C for 24 h. Iodine solution was flooded on the starch agar plates for 30 s after 24 h incubation. Presence of clear zone around the growth of isolates were considered as amylase producers and sub-cultured on starch agar slants for further investigation.

Amylase production by using submerged state fermentation

A mineral broth medium (peptone 6 g/L; MgSO₄ 0.5 g/L; KCl 0.5 g/L and starch 1 g/L) was prepared. From the broth medium, 90 mL was transferred into 150 mLcapacity Erlenmeyer flasks and sterilized at 121°C for 15 min. A loopful of inoculum was transferred into five test tubes having a 10 mL of sterile nutrient broth. The test tubes were incubated at 37°C for 24 h until the visible turbidity and density becomes equal to 0.5 McFarland standards (1x10⁵ CFU/mL). Then after, 2 mL suspension of the isolates was taken from overnight cultures of test tube and inoculated into 90 mL of flasks and incubated in a water bath by adjusting the temperature at 25, 30, 35, 37, and 40°C for 24, 48, 72 and 96 h under the rotary shaker by the speed of 150 rpm. Finally, the fermented culture was poured into centrifuge tubes, spin for 20 min at 5000 rpm and extracted by decantation method (Aiyer, 2005).

Effect of substrate concentration and fermentation time

The effect of substrate concentration was determined by using different concentrations of starch (1.0, 2.0, 3.0, 4.0 and 5.0%) in the amylase production medium. The effect of fermentation time was also determined by incubating the amylase production medium at different fermentation time (24, 48, 72 and 96 h).

Effect of pH and temperature

The effect of pH and temperature on amylase activity was confirmed by adjusting the pH value of the fermentation medium at 4.0, 5.0, 6.0, 7.0, 8.0, 9.0 and the temperature at 25, 30, 35, 37 and 40°C respectively.

Characterization and identification of amylase producing isolates

Cultural, morphological and biochemical characterization of isolates

The isolates were observed macroscopically and microscopically to characterize the colony morphology and gram reaction respectively. In addition, isolates were also characterized biochemically by different biochemical tests such as Simmons citrate, Urease, Methyl Red/Voges Proskauer (MR/VP) and Starch hydrolysis tests.

RESULTS AND DISCUSSION

Isolation and primary screening of amylase producing bacteria and actinomycetes

A total of 18 isolates were obtained from the collected soil samples and coded as Sp1-Sp18 based on their maximum clear zone respectively. Of the 18 isolates, five isolates showed higher clear zone after flooding with iodine solution (Table 1) and this result agreed with the report of Hmidet et al. (2009) and only a few selected strains of bacteria from soil sample were obtained. According to Bergey's Manual of Determinative Bacteriology, isolates were grouped into two genera; namely, genus *Bacillus* (Sp1, Sp3, Sp4 and Sp5) and genus *Streptomyces* (Sp2). As per the primary screening of the isolates, these two genera could be potential candidates for several industrial applications which were agreed by the report of Ashwini et al. (2011).

The five isolates were characterized by cultural and microscopic methods to differentiate their respective genera. Most of the isolates have shown a regular form,
Table 1. Isolates and their clear zone on starch agar plates during primary screening.

<table>
<thead>
<tr>
<th>Isolates</th>
<th>Clear zone (mm)</th>
<th>Isolates</th>
<th>Clear zone (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sp1*</td>
<td>22 ± 0.20</td>
<td>Sp10</td>
<td>8 ± 0.01</td>
</tr>
<tr>
<td>Sp2*</td>
<td>20 ± 0.50</td>
<td>Sp11</td>
<td>7 ± 0.90</td>
</tr>
<tr>
<td>Sp3*</td>
<td>19 ± 0.20</td>
<td>Sp12</td>
<td>6 ± 0.55</td>
</tr>
<tr>
<td>Sp4*</td>
<td>18 ± 0.60</td>
<td>Sp13</td>
<td>6 ± 0.32</td>
</tr>
<tr>
<td>Sp5*</td>
<td>18 ± 0.10</td>
<td>Sp14</td>
<td>5 ± 0.45</td>
</tr>
<tr>
<td>Sp6</td>
<td>11 ± 0.40</td>
<td>Sp15</td>
<td>5 ± 0.31</td>
</tr>
<tr>
<td>Sp7</td>
<td>10 ± 0.70</td>
<td>Sp16</td>
<td>5 ± 0.56</td>
</tr>
<tr>
<td>Sp8</td>
<td>10 ± 0.90</td>
<td>Sp17</td>
<td>4 ± 0.11</td>
</tr>
<tr>
<td>Sp9</td>
<td>10 ± 1.00</td>
<td>Sp18</td>
<td>3 ± 0.22</td>
</tr>
</tbody>
</table>

Mean ± Standard deviation of triplicate determination for primary screening, *Isolates selected for amylase production.

Table 2. Cultural and microscopic characteristics of the five isolates.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sp1</td>
</tr>
<tr>
<td>Form</td>
<td>Regular</td>
</tr>
<tr>
<td>Color</td>
<td>Creamy</td>
</tr>
<tr>
<td>Gram staining</td>
<td>Positive</td>
</tr>
<tr>
<td>Shape</td>
<td>Rod</td>
</tr>
</tbody>
</table>

Table 3. Biochemical characteristics of isolates.

<table>
<thead>
<tr>
<th>Biochemical characters</th>
<th>Isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td>Starch hydrolysis test</td>
<td>Sp1</td>
</tr>
<tr>
<td>Urease test</td>
<td>+</td>
</tr>
<tr>
<td>Simon’s Citrate test</td>
<td>+</td>
</tr>
<tr>
<td>Methyl Red/Voges Proskauer test</td>
<td>+/-</td>
</tr>
<tr>
<td>Indole test</td>
<td>+</td>
</tr>
</tbody>
</table>

+ Positive, - Negative.

creamy color and rod shape of colony morphology (Table 2).

Biochemical characterization

Based on the biochemical tests, all the isolates showed positive results of starch hydrolysis, Urease, MR/VP and indole tests (Table 3).

Effect of fermentation time on amylase activity

All the isolates were showed maximum amylase activity at 48 h of submerged fermentation time and then declined (Figure 1). Similar findings were also observed on Bacillus subtilis and Bacillus sp. DLB9 (Shyam et al., 2013). The reason for amylase activity decrement after 48 h might be due to the suppression and accumulation of other by-products in the fermentation medium and also depletion of nutrients as reported by other studies (Haq et al., 2010).

Effect of starch concentration on amylase activity

In general, amylase activity was increased with the increment of starch concentration from 1 to 4%. In this study, highest amylase activity was observed at 4% starch concentration (Figure 2). If the starch concentration
goes beyond 4% amylase activity was declined. This might be associated with metabolizing capacity of the isolates within the short period of time when the starch concentration was increased. The present findings are in corroborating with the report of earlier findings on amylase activity obtained from Bacillus species (Oyeleke and Oduwole, 2009).

**Effect of pH on amylase activity**

In this study, highest amylase activity was observed at neutral pH. These results was also in agreement with the previous report for amylase activity of Bacillus strains such as B. thermooleovorans NP54, B. coagulans, B. licheniformis, and B. subtilis JS-2004 within the range of 6-7 pH (Gupta et al., 2010; Mendu et al., 2005; Adeyanju et al., 2007; Mrudula and Kokila, 2009). This implies that capability of the amylase activity within the neutral pH might be due to the fact that the isolates were inactive in the acidic or alkali medium. Different microorganisms have different optimum pH; if any variation on pH value results in poor microbial growth and amylase activity (Lonsane and Ramesh, 2009; Pandey et al., 2000).
Effect of temperature on amylase activity

Temperature is one of the environmental factors for amylase production which is usually varied from one organism to another. For example, *Bacillus amyloliquefaciens*, *B. subtilis*, *B. licheniformis* and *B. stearothermophilus* are some of the commonly used *Bacillus* sp. reported to produce amylase at 37-60°C (Asgher et al., 2007). In the present study, maximum amylase activity was observed at 40°C (Figure 4). The result also showed a positive correlation between the amylase activity and the incubation temperature up to 40°C, followed by a gradual decrease. At higher temperature, bacterial growth gets suppressed and consequently amylase activity was also inhibited (Oyeleke and Oduwole, 2009). The isolates grown well and revealed high amylase activity in the temperature range from 35 to 40°C. However, maximum amylase activity of *Bacillus* was 40°C and *Streptomycyes* at 37°C. Mishra and Behera (2008) also reported that most of the bacterial isolates were produced and showed amylase...
activity at elevated temperature in particular amylase activity of *Bacillus* species at the range of 40-45°C.

**Conclusion**

Based on the present findings, it is concluded that the soil is a potential source for amylase producing microorganisms, which could be exploited for the production of important industrial amylase. The results also showed that there was appreciable high amylase production from the isolates under optimized conditions of fermentation time, temperature, pH and starch concentration. *Bacillus* was found to be most frequently occurring amylolytic bacteria followed by *Streptomyces*.

**Conflict of interests**

The author declared that there is no conflict of interest.

**ACKNOWLEDGEMENT**

The author acknowledged the Department of Biology, College of Natural and Computational Sciences, University of Gondar, Ethiopia for giving laboratory facilities to perform this research work.

**REFERENCES**


Short Communication

Action of proteases of the nematophagous fungi *Pochonia chlamydosporia* on *Ascaris suum* eggs of collared peccary (*Pecari tajacu*)

Filippe Elias de Freitas Soares†, José Humberto de Queiroz†, Jackson Victor de Araújo‡, Maria Gorete Ramos Rodrigues§, Alexandre de Oliveira Tavela‡, Anderson Rocha Aguiar§, Tracy Lacerda§, Carolina Magri Ferraz§, Maria Cristina Valdetaro Rangel§, Thiago Senna§, Andreia Luíza Araújo§, Tarcízio de Paula Rego‡, Caio Colodette Sena§ and Fabio Ribeiro Braga§

†Departamento de Bioquímica e Biologia Molecular. Universidade Federal de Viçosa, Viçosa-MG. Cep: 36570000, Brasil.
‡Departamento de Veterinária, Universidade Federal de Viçosa, Viçosa-MG. Cep: 3657000, Brasil.

Received 29 June, 2015; Accepted 3 August, 2015

Among the parasites of domestic and wild swine, *Ascaris suum* stands out; a nematode that can lead to growth retardation and reduction in weight gain due to its action, especially in young animals. The objective of this study was to test the ovicidal action of proteases from *Pochonia chlamydosporia* (VC4) on *A. suum* eggs in an assay with Petri dishes. The fungus *P. chlamydosporia* (VC4) was grown in Erlenmeyers flasks with 50 ml of liquid minimal media supplemented with 0.2% gelatin for production of enzymes. In the present assay, 500 eggs were poured into Petri dishes of 4.5 cm in diameter and 5 ml of VC4 proteases were added in each Petri dish and incubated at 26°C in the dark for 14 days. After this period, the number of embryonated and destroyed *A. suum* eggs present in each plate from treated and control groups was counted. Significant difference (*p* <0.01) was found between the number of eggs from treated group compared to the control group. At the end of the experiment, the proteases of *P. chlamydosporia* (VC4) demonstrated efficacy in reducing embryonated eggs on the plates of the treated group (78.7%) compared to the control group (83.7%). The results presented in this study demonstrate that proteases of *P. chlamydosporia* (VC4) were effective in the destruction of *A. suum* eggs and therefore could be used as biological control of this nematode.

Key words: Nematophagous fungi, *Pochonia chlamydosporia*, protease, *Pecari tajacu*, *Ascaris suum*.

INTRODUCTION

The commercial breeding of wild animals in several countries has been identified as an important source of protein used to the livelihoods of the poorest people living in the countryside. However, there are some obstacles
for the commercial production of these animals, and, among these, the gastrointestinal nematodiosis, that deserve attention (Bonuti et al., 2002). Among the parasites of domestic and wild swine, *Ascaris suum* stands out, a nematode that can lead to growth retardation and reduction in weight gain due to its action, especially in young animals (Urquhart et al., 1998). Worn control in animals is carried out with the use of anthelmintic drugs, however, these drugs may not be fully effective mainly due to the parasitic resistance (Bowman et al., 2006). Thus, alternative measures that may help to decrease the continued use of a same class of anthelmintic, as well as the use of doses higher than recommended, are required, and among these alternatives is the biological control conducted with natural antagonists of nematodes, with emphasis on the nematophagous fungi (Braga and Araújo, 2014). In the environment, these fungi are biologically very important because they play a role in the recycling of carbon, nitrogen and other elements that originate from the degradation of nematodes (Braga et al., 2007).

Nematophagous fungi are the major natural antagonists of nematodes in the environment. They are divided into predators, endoparasites and ovicides. In the group of ovicidal fungi, the species *Pochonia chlamydosporia* stands out (Araújo et al., 2008; Braga and Araújo, 2014). These fungi secrete extracellular enzymes from the class of proteases, which develop an important role in infection and destruction (ovicidal activity) of eggs of the nematodes (Yang et al., 2013; Khan et al., 2004). On the other hand, the ovicidal action of *P. chlamydosporia* (VC4) and its proteases has been successfully tested against eggs from various genera of helminth under laboratory conditions (Braga et al., 2008a, b, 2009a; Soares et al., 2014). However, this isolate had never their proteases tested on eggs of *A. suum* of a wild swine, as *Pecari tajacu*.

The objective of this study was to test the action of proteases of *P. chlamydosporia* (VC4) on eggs of *A. suum*.

### MATERIALS AND METHODS

Fungus

One isolate of ovicidal fungus *P. chlamydosporia* (VC4) from mycology collection of the Parasitology Laboratory of the Department of the Federal University of Viçosa Veterinary, Minas Gerais, Brazil was maintained in test tubes containing 2% CMA and corn-meal-agar (2% CMA) in the dark for 10 days.

Culture dishes of 4 mm in diameter were extracted from fungal cultures maintained in test tubes containing 2% CMA and transferred to Petri dishes of 9.0 cm in diameter containing 20 mL of 2% potato dextrose agar (2% PDA), maintained at 26°C in the dark and during 10 days. After the growth of the isolate, novel culture dishes of 4 mm in diameter were transferred to Petri dishes of 9.0 cm in diameter containing 20 mL of 2% water-agar (2% WA), maintained at 26°C in the dark and during 10 days (Araújo et al., 2008).

### Protease production of *P. chlamydosporia* (VC4)

*P. chlamydosporia* (VC4) was cultured in flasks vials with 50 mL of liquid medium (0.3 g/L NaCl, 0.3 g/L MgSO₄.7H₂O, 0.3 g/L K₃HPO₄, 0.2 g/L yeast extract) supplemented with 0.2% gelatin. Gelatin was filtered through Millipore filter (with 45 μm aperture) before being added aseptically in autoclaved medium. The samples containing the isolate were incubated in the dark at 28°C in a rotary shaker at 120 rpm. After five days, the supernatant was collected and filtered using Whatman filter paper No. 1 at 4°C according to Esteves et al. (2009).

### Obtaining of *Ascaris suum* eggs

*A. suum* eggs were recovered from the dissection of adult specimens, obtained during the necropsy of a collared peccary (*P. tajacu*), who died under natural conditions. The identification of adult parasites followed the standards described by Soulsby (1982). Subsequently, the eggs were analyzed for their integrity under a light microscope in 10x objective according to Urquhart et al. (1998).

### Experimental assays

**Enzymatic assay**

An *in vitro* assay was performed to confirm the protease activity of the fungus *P. chlamydosporia* (VC4), where the protease activity was measured as described by Soares et al. (2014) modified. The volumes of the solutions used in this method were: 100 μL of proteases, 400 μL of Tris-HCl 100 mM (pH 7.0) buffer and 500 μL of 1% casein pH 8.0. The reaction medium was incubated for 60 min and the reaction stopped by adding 1 mL of 10% trichloroacetic acid solution. After 10 min, the reaction medium was centrifuged at 10,000 x g for 5 min, the supernatant collected and the absorbance determined spectrophotometrically at 280 nm. A standard curve was constructed varying the concentrations of tyrosine (15 to 200 μg/mL). One protease unit was defined as the amount of enzyme required to liberate 1.0 μg of tyrosine per minute under the assay conditions used. The assay was performed in triplicate.

**Ovicidal assay**

Five hundred *A. suum* eggs were transferred into Petri dishes of 4.5 cm in diameter. Then there was added 5 mL of proteases from *P. chlamydosporia* (VC4) to each Petri dish in the treated group which was sealed with Rolapack film and incubated at 26°C, in the dark, for 14 days. The control group contained 500 *A. suum* eggs in 10 mL of denatured enzymes in Petri dishes, which were incubated under the same conditions. Six replicates were performed for each group. After 14 days, the number of eggs from *A. suum* present in each Petri dish from treated and control groups was calculated according to the method described by Soares et al. (2014) and Mukhtar and Pervaz (2003). The eggs were counted by means of light microscopy. Data obtained in the experimental test were subjected to analysis of variance at significance levels of 1 and 5% probability and to non-parametric Friedman test with 1% probability (Ayres et al., 2003). The average reduction percentage of *A. suum* eggs was calculated according to the following equation:

\[
\text{%Reduction} = \left(\frac{\text{Average of eggs from control} - \text{Average of eggs from treatment}}{\text{Average of eggs from control}}\right) \times 100
\]
RESULTS

The proteases of *P. chlamydosporia* (VC4) showed ovicidal activity (destruction of eggs) in Petri plates from treated group after 14 days of interaction (Figure 1). However, destroyed eggs were not observed in the plates of the control group after the same time interval. On the other hand, the reduction percentage of 75% of the *A. suum* eggs in the treated group was observed compared to the control group. Additionally, the protease activity of the fungus *P. chlamydosporia* (VC4) was confirmed and measured in the *in vitro* assay. The value of the proteolytic activity was 9.38 U/mL, with a standard deviation of 0.47 U/mL.

Significant difference (p < 0.01) was found between the number of destroyed eggs (ovicidal activity) on the plates of the treated group compared to the control group. Furthermore, regarding the percentage of non-embryonated eggs in plates from treated group, the proteases of *P. chlamydosporia* (VC4) were effective in relation to the control group. The percentage of non-embryonated eggs were 78.7 and 83.5% for the treated and control groups, respectively.

DISCUSSION

In the present study, we demonstrated the enzymatic activity of proteases of *P. chlamydosporia* (VC4) in the destruction of *A. suum* eggs at the end of 14 days of interaction. This result is in agreement with those of Esteves et al. (2009), who worked with *P. chlamydosporia* grown in liquid medium supplemented with 0.2% gelatin and then demonstrated its action on hatching of *Meloidogyne* spp. eggs, a phytonematode. In addition, the proteolytic activity of the fungus *P. chlamydosporia* (VC4) was measured in order to confirm its enzymatic action. An activity value of 9.38 U/mL was observed; this value being similar to that found by Braga et al. (2012) using the same fungus. Nevertheless, in relation to the ovicidal activity of *P. chlamydosporia* on eggs from *Ascaris* genus, some studies have been conducted with this fungus in experimental assays in 2% solid water-agar (2% WA). Braga et al. (2007) showed that *P. chlamydosporia* (VC4) was effective in the destruction of *A. lumbricoides* eggs under laboratorial conditions, noting at the end of the experiment, a percentage of 26% in the destruction of eggs. In another study, Araújo et al. (2008) demonstrated the efficacy of the same fungus in the destruction of *A. suum* eggs in three day intervals (7, 14 and 21 days), and that at the end of 14 days, they reported 17.7% percentage of eggs destroyed. Furthermore, these studies have not studied the action of the fungus on the embryonation of eggs. In this study, we proved the destruction of the *A. suum* eggs by proteases of *P. chlamydosporia* (VC4) and its effectiveness in embryonation thereof, with 75% reduction percentage.

Braga et al. (2009b) reported that *P. chlamydosporia* (VC4) had proven efficacy in destroying *Austroxyuris finlaysoni* eggs, one oxyuridae of marsupials, in 2% WA. In that work, a percentage of 21.0% of eggs destroyed was registered after 15 days. This information is interesting because in that paper the authors discuss the difficulty of controlling worms in wild animals. However, the authors of this study mention by means of the results obtained a new verminosis control alternative in wild animals kept in zoos and in commercial breeding.

In several countries, the *Ascaris* genus has been mentioned in wild swine bred in captivity, causing abdominal
cramps and intestinal obstruction in these animals (Carlos et al., 2008). Furthermore, Mundim et al. (2004), reported that roundworms are very frequent and their eggs have long period of resistance in the environment, facilitating the infection of the animals and contributing to its high frequency. In this context, by means of the results obtained in this study, the employability of *P. chlamydosporia* and its proteases as an alternative of environmental control of *A. suum* eggs from wild swine bred in captivity (zoos and commercial breeding) is suggested. Moreover, this is the first report of the action of these enzymes from *P. chlamydosporia* on *A. suum* eggs of a wild swine (*P. tajacu*). However, further studies on these proteases will be the focus of other works.

**Conflict of interests**

The authors did not declare any conflict of interest.

**ACKNOWLEDGEMENTS**

The authors thank Fapemig, CNPq and CAPES / FINEP for financial support.

**REFERENCES**


African Journal of Microbiology Research

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