OPEN ACCESS

May 2022
ISSN 1684-5315
DOI: 10.5897/AJB
www.academicjournals.org

ACADEMIC JOURNALS
expand your knowledge

About AJB

The African Journal of Biotechnology (AJB) is a peer reviewed journal which commenced publication in 2002. AJB publishes articles from all areas of biotechnology including medical and pharmaceutical biotechnology, molecular diagnostics, applied biochemistry, industrial microbiology, molecular biology, bioinformatics, genomics and proteomics, transcriptomics and genome editing, food and agricultural technologies, and metabolic engineering. Manuscripts on economic and ethical issues relating to biotechnology research are also considered.

Indexing

CAB Abstracts, CABI's Global Health Database, Chemical Abstracts (CAS Source Index) Dimensions Database, Google Scholar, Matrix of Information for The Analysis of Journals (MIAR), Microsoft Academic, Research Gate

Open Access Policy

Open Access is a publication model that enables the dissemination of research articles to the global community without restriction through the internet. All articles published under open access can be accessed by anyone with internet connection.

The African Journals of Biotechnology is an Open Access journal. Abstracts and full texts of all articles published in this journal are freely accessible to everyone immediately after publication without any form of restriction.

Article License

All articles published by African Journal of Biotechnology are licensed under the Creative Commons Attribution 4.0 International License. This permits anyone to copy, redistribute, remix, transmit and adapt the work provided the original work and source is appropriately cited. Citation should include the article DOI. The article license is displayed on the abstract page the following statement:

Memberships and Standards

Academic Journals strongly supports the Open Access initiative. Abstracts and full texts of all articles published by Academic Journals are freely accessible to everyone immediately after publication.

All articles published by Academic Journals are licensed under the Creative Commons Attribution 4.0 International License (CC BY 4.0). This permits anyone to copy, redistribute, remix, transmit and adapt the work provided the original work and source is appropriately cited.

Crossref is an association of scholarly publishers that developed Digital Object Identification (DOI) system for the unique identification published materials. Academic Journals is a member of Crossref and uses the DOI system. All articles published by Academic Journals are issued DOI.

Similarity Check powered by iThenticate is an initiative started by CrossRef to help its members actively engage in efforts to prevent scholarly and professional plagiarism. Academic Journals is a member of Similarity Check.

CrossRef Cited-by Linking (formerly Forward Linking) is a service that allows you to discover how your publications are being cited and to incorporate that information into your online publication platform. Academic Journals is a member of CrossRef Cited-by.

Academic Journals is a member of the International Digital Publishing Forum (IDPF). The IDPF is the global trade and standards organization dedicated to the development and promotion of electronic publishing and content consumption.

Contact

Editorial Office:	ajb@academicjournals.org
Help Desk:	helpdesk@academicjournals.org
Website:	http://www.academicjournals.org/journal/AJB
Submit manuscript online	http://ms.academicjournals.org

Academic Journals
73023 Victoria Island, Lagos, Nigeria
ICEA Building, 17th Floor,
Kenyatta Avenue, Nairobi, Kenya.

Editor-in-Chief

Editorial Board Members

Table of Content

Commercialization of genetically modified crops in Africa: Opportunities and challenges 197

Endale Gebre Kedisso, Karim Maredia, Joseph Guenthner and Muffy Koch

Preliminary verification of the adoption status of some yam (*Dioscorea rotundata* and *Dioscorea alata*) varieties in Nigeria using microsatellites markers 207

Nwogha J. S., J. E. Obidiegwu, R. N Okereke, R. Bhattacharjee and H. O. Oselebe

Efficient callus-mediated system for commercial production of sugarcane (*Saccharum* spp.) planting material in Ghana 217

Elaine Azu, Wilfred Elegba, Abigail Tweneboah Asare, Kwame Asare, Christian Akama, Patience Asare, Clement Annor, Samuel Azure and Kenneth Danso

Characterization and antimicrobial activities of lactic acid bacteria isolated from selected Nigerian traditional fermented foods 236

Chinedu Godspower Ohaegbu, Anayochukwu Chibuike Ngene, Unyime Inyang Asuquo, Oluwatoyin Debby Coulthard and Ejikeme Nwachukwu

New technique for improving fish packaging hygiene and prolonged shelf life 251

Nagwa T. Elsharawy, Wafa A. Baabdullah and Ali Alkaladi

Vol. 21(5), pp.188-197, May 2022
DOI: 10.5897/AJB2021.17434
Article Number: A458D8168996
ISSN: 1684-5315

http://www.academicjournals.org/AJB

ACADEMIC JOURNALS
expand your knowledge

African Journal of Biotechnology

Full Length Research Paper

Commercialization of genetically modified crops in Africa: Opportunities and challenges

Endale Gebre Kedisso[1*], Karim Maredia[1], Joseph Guenthner[2] and Muffy Koch[3]

[1]College of Agriculture and Natural Resources, Michigan State University, East Lansing, Michigan, USA.
[2]Agricultural Economics and Rural Sociology, College of Agricultural and Life Sciences, University of Idaho, Moscow, Idaho, USA.
[3]J. R. Simplot Company, Boise, Idaho, USA.

Received 24 November, 2021; Accepted 8 February, 2022

Genetically modified (GM) crops offer potential for enhancing agricultural productivity for smallholder farmers in Africa. After nearly three decades of research and development collaboration and regulatory capacity strengthening, several countries in Sub-Saharan Africa (SSA) are moving towards commercializing GM crops for the benefit of smallholder farmers. South Africa approved genetically modified (GM) cotton, maize and soybeans in the 1990s. Nigeria, Ethiopia, Kenya, Sudan, Eswatini and Malawi recently approved general release of GM crops, including GM cotton, GM cowpea, GM maize, and GM cassava through public-private partnerships. Collected data from a diverse group of 30 stakeholders from 14 countries in Africa and results indicated that while progress has been made towards commercializing GM crops in several countries in Africa, some key challenges and downstream issues remain to be addressed. These include building functional regulatory systems, vibrant seed systems, local seed production, effective extension services, reliable credit/financial and marketing services, and improved access to markets for smallholder farmers. Unless these downstream issues are effectively addressed, smallholder farmers in Africa will not benefit from GM crops.

Key words: Agricultural Biotechnology, genetically modified crops, commercialization, technology transfer, technology deployment, Africa.

INTRODUCTION

Advances in modern biotechnology such as genetic modification and genome editing offer new opportunities for improving crops and enhancing agricultural productivity worldwide. In 2018, about 17 million farmers, 95% of whom are smallholder farmers, planted 190.7 million hectares of GM crops globally (Noisette, 2021). In Africa, several countries are now commercializing GM crops that are appropriate for African farmers and farming constraints. South Africa was the first African country to enact a regulatory framework that allowed GM crop cultivation, import and export. South Africa is the largest GM crop producer (2.7 million ha) in Africa, followed by

*Corresponding author. E-mail: kedissoe@msu.edu. Tel (Cell): +15174810999.

Sudan (243,000 ha), Ethiopia (1,300 ha in 2021), Eswatini (250 ha) and Kenya (1500 ha in 2021) (Silas et al., 2022; USDA, 2020; ISAAA, 2018). To date, the governments of 12 countries in SSA have a functional biosafety regulatory framework. The governments of seven countries - Ethiopia, Eswatini, Kenya, Malawi, Nigeria, South Africa and Sudan - have approved the general release of GM crops (Turnbull et al., 2021; Gbashi et al., 2021).

Currently, there are 13 GM crops in different stages of research and development in 13 African countries, with 13 different traits (ISAAA, 2018). Cotton (*Gossypium* spp.), maize (*Zea mays*), cowpea (*Vigna sinensis*), rice (*Oryza sativa*), sorghum (*Sorghum bicolor*), potato (*Solanum tuberosum*), sweet potato (*Ipomea batatus*), cassava (*Manihot esculenta*), banana (*Musa sapientum*) and sugarcane (*Saccharum officinarum*) are either being tested in confined field trials (CFT) or are approved for general release (Akinbo et al., 2021). Since 2018, four countries have initiated commercial production after approval for general release (ISAAA, 2020): Ethiopia (Bt-cotton), Malawi (Bt cotton), Kenya (Bt cotton) and Nigeria (Bt cotton and insect resistant cowpea). Burkina Faso and Egypt, both earlier adopters of GM crops, faced challenges after commercialization and suspended production. In Burkina Faso, Bt cotton commercial production was suspended in 2016 due to fiber quality. Egypt suspended production of GM maize through a Ministerial decree in 2012 (Turnbull et al., 2021) over concerns about trade with the EU.

Overall, close to three million hectares of GM crops were planted across Africa in 2021. Despite considerable global advancement of GM crops, progress in commercialization in Africa has been slower than expected (Gbashi et al., 2021; Azadi et al., 2015). After nearly three decades of safe use of GM crops and documented benefits for smallholder farmers (Kouser and Qiam, 2011; Bennett et al., 2006), governments of many African countries are still debating and delaying the use of GM crops.

Numerous reports from health and environmental safety research have confirmed the safety and benefits of GM crops and their derived products (Gbashi et al., 2021; Oloo et al., 2020; Bayer Crop Science, 2020; EU Report, 2010; ISAAA Biotech Update, 2010). Such scientific evidence has not removed lingering public perception and controversies about the environmental and food safety concerns of GM crops (Mbabazi et al., 2016).

Public safety concerns have limited GM crop development, and there are many other barriers to commercialization of already approved products. After CFT, which focus on event selection and regulatory data collection, GM cultivars, like conventional cultivars, are subject to further evaluation through national performance trials (NPTs) and verification studies to meet national cultivar release requirements (Akinbo et al., 2021). National research programs, biosafety authorities and other regulatory agencies work in parallel to evaluate safety and performance results, complete risk assessments, release and register GM crops.

After safety approval and cultivar registration, the roles of regulators diminish and commercialization processes become the focus. The private sector, seed systems, extension systems and business methods function to deploy new GM crops and this transition to commercial deployment is complex and rarely smooth. In Africa, commercialization needs to take into account the prevalence of an informal seed sector, the importance and needs of smallholder farmers, the role of the public sector in producing and supplying improved planting material and extension services, and how the private sector will work alongside these systems. Additional commercialization responsibilities for GM crops must be addressed, including the promotion of new products, obtaining wider acceptance for GM technology, ensuring farmer access to sustainable new products, maintaining product quality and responsible introduction through stewardship.

Roadmap for commercialization of GM crops

Since the first field trial of a GM product in South Africa in 1989, progress has been made on the adoption of biotechnology products. In Africa, the number of laboratory and field trials for product development has increased reflecting scientific optimism for the benefits of the GM crops in the midst of prolonged controversy surrounding modern biotechnology (Waithaka et al., 2015). Along with product development and commercialization, product stewardship is implemented across the process to support the responsible release of safe, innovative GM products (ETS, 2017).

Bringing a conventional or GM crop cultivar to market requires many years of systematic development, testing, and selection. Private and public sector developers apply stepwise review and decision-making processes to monitor the development of new products and to ensure that only good events are moved through to commercialization and into the hands of farmers. These roadmaps for product development and commercialization ensure that important considerations are investigated at each step of development (Cooper, 2006). Re-evaluation takes place at the end of each development step for each product. This reevaluation includes a review of product performance, business fit with existing strategy goals, marketing plans, intellectual property management and licensing, regulatory requirements, product stewardship, and the commercialization strategy.

Input on each of these areas informs the Yes/No decisions that are essential for effective product

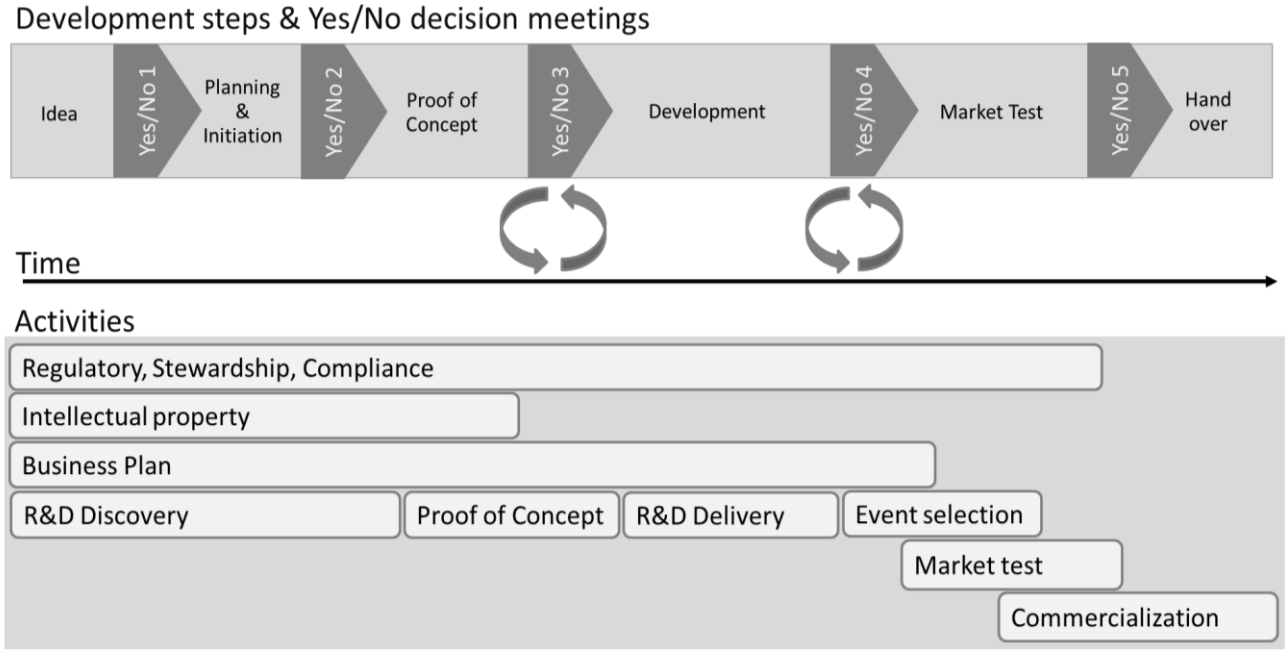

Figure 1. Outline of a product commercialization process for a GM plant cultivar.

development (Figure 1). At all the decision meetings, the product data are reviewed and a decision is made on whether to continue or halt the development of the product. Sometimes a decision to continue may include a step back to repeat and address certain activities that will help ensure the quality needed for a successful product.

Successful commercialization of a GM cultivar requires a well-planned strategy with input from experts in a wide range of fields such as agronomy, molecular biology, entomology, pathology, plant transformation, biochemistry, food science, legal, regulatory, stewardship, seed production, communication, business, marketing, economics, environmental sciences and social sciences. Coordination of the steps and expert input requires effective project management and clear criteria for the decisions that are taken at each step in the development process. Missing one area of expert input or indecisive decision-making at any one stage can lead to costly oversights and a considerable waste of money and time. In general, the process outlined in Figure 1 takes 10 to 15 years and can cost upwards of US$ 50 million for a new GM cultivar depending on the crop, traits, technology, markets, and efficiency of the regulatory approval processes.

Since GM crop commercialization in the mid-1990s, enhanced capacity for regulatory compliance and product stewardship became critical to help ensure safe and sustainable use of biotechnology products. Effort was made in some countries to establish research and development capacity, which are lacking in most countries in Africa. Stakeholders have identified several factors causing delay to commercialization of GM crops, including socio-economic constraints, high cost of technology or seed, fear of corporate monopolies contrasting with weak private sector involvement, and inadequate awareness of best practices for commercial release of new planting material (Mbabazi et al., 2020; NASAC, 2015, in preparation). The need for stronger public-private partnerships in research, product development and product commercialization has emerged as a key success factor for commercializing GM crops in developing countries (NASAC, 2015).

Weak seed systems and weak financial/credit systems can also limit farmer access to technology and new products. A recent study on Bt cotton hybrid seed access by African farmers indicated that weak coordination among various stakeholders along the seed value chain exacerbated the problem of sustainable supply and hindered wider utilization of the approved GM crops (Mbabazi et al., 2020; Alhassan et al., 2018). Stakeholder lack of awareness, inadequate demonstration of new technology to farmers and poor handling of the new technology by farmers, including poor extension systems all contributed to slow commercialization of improved planting material (Turnbull et al., 2021; Mbabazi et al., 2020). More efforts are needed to establish partnerships at national, regional, and international level to bridge the knowledge gaps in research, regulation, extension, commercialization, communication, marketing and trade (NASAC, 2015).

Table 1. Participants of biotechnology and biosafety and food safety courses who were surveyed for this paper.

Country and participants (number)	Affiliations[1]			
	Regulatory	Academic	Research	NGO
Burkina Faso	2			
Cameroon	1			
Cote D'Ivoire		1		
Eswatini	4	2	2	
Ethiopia	7		4	
Ghana	15			2
Kenya	2			2
Mali	1			
Mozambique	3			
Niger			2	
Nigeria	7			
Rwanda	3			
Senegal	3			
Togo		3	1	
USDA-FAS[2]	7 participants from USDA in 7 countries			

[1] "Regulatory" includes regulators, policy makers and lawyers.[2] USDA-FAS = United States Department of Agriculture, Foreign Agriculture Service.

This paper reports on stakeholder assessment of the support needed for successful access to and utilization of GM crops in Africa.

METHODS

We collected four sets of data from 74 African stakeholders (including 7 USDA country representatives) that attended two Michigan State University (MSU) international short courses in 2021: (1) a pre-course survey using semi-structured questionnaire, (2) an 'end of course' evaluation, and (3) in-course enquiry from course 1, and (4) in-course enquiry from course 2. The two courses were on agricultural biotechnology and biosafety and food safety. Countries represented and number of participants is shown in Table 1. In the two courses, participants represented a diverse group of stakeholders including regulators, policymakers, scientists, academic specialists, lawyers and representatives of non-governmental organizations. The questionnaire was distributed prior to the start of the course to 30 participants of the first course for participants representing 14 countries from Africa: Burkina Faso, Cameroon, Cote D'Ivoire, Eswatini, Ethiopia, Ghana, Kenya, Mali, Mozambique, Niger, Nigeria, Rwanda, Senegal and Togo. The survey included questions on biotechnology, biosafety, regulatory needs and commercialization of biotech crops.

The survey questionnaire had 100 questions containing multiple choice answering options where respondents replied choosing "Not important", "Somewhat important", "Very important"; or "Strongly disagree", "Somewhat agree", "Strongly agree" based on the situation in their respective countries. Specific questions were related to challenges, concerns, commercialization, public perceptions, personal experiences and other issues pertaining to biotechnology and biosafety.

The pre-assessment survey questionnaire was supplemented with written and oral enquiries and end of course evaluation questions (160 questions) raised by stakeholders that attended the short courses. The questions were recorded and categorized into representative themes: product development, regulation, technology transfer (including IP, licensing, scaling up, seed systems), communication and outreach, public acceptance and trade to understand stakeholders areas of concern. The information included in this paper is part of a needs assessment survey on biotechnology and biosafety development, level of awareness of advances in the biotech product commercialization and genome editing technologies in developing countries, as well as the challenges faced and capacity building needs for commercialization and adoption of GM crops.

RESULTS

The survey assessed stakeholders experience with GM products and found that 96% of the 30 respondents have some level of awareness about GM crops. Most of the respondents (84%) had some level of awareness about genome editing (Table 2). About 54% recognized they had consumed food containing GM products and a third (35%) did not know if they had ever consumed any GM food product. Asked if there is a delay in commercialization of GM crops, most agreed (85%) that there was an undue delay in commercialization of useful GM crops in their countries. The results reflect the high anticipation of stakeholders for biotech progress in their home countries. Stakeholders also showed optimism towards improving public perception and attitude towards GM crops.

Stakeholders' understanding of issues that delay wider

Table 2. Stakeholder awareness of agriculture R&D and their assessment of public perception towards GM crops in Africa.

Attribute	Responses		
	Yes (%)	No (%)	Don't know (%)
Engagement in agriculture R&D	68	32	0
Awareness of GM crops	96	4	0
Awareness of genome edited crops	84	16	0
Consumed food containing GM content	54	12	35
Presence of delay in GM crop adoption	85	15	0

Table 3. Stakeholder perception of issues that delay adoption of GM crops in Africa.

Issues causing delay	Strongly agree (%)	Somewhat agree (%)	Strongly disagree (%)
Lack of access and availability of new technology	82	9	9
Lack of information	81	5	14
Lack of marketing	77	18	0
Lack of political will	68	18	9
Food safety concerns	67	19	0
Cost of technology/seeds	65	15	5
Poor regulation	59	13	18
Environmental safety	59	23	9
Absence of distributors	57	24	10
Lobbying by GM opponents	52	14	10
Trade concerns and loss of market access	38	29	10
Farmers lack of interest	27	27	46
Technology not promising	25	20	35

adoption of GM crops is an important consideration of the assessment (Table 3). Most stakeholders agreed that lack of access and availability of GM crops (82%) and lack of information and awareness (81%) contributed most to delayed adoption of GM crops. Other issues such as political will (68%), safety concerns (67%) and costs of seeds (65%) are among the key bottlenecks that participants agree are challenging expanded use of GM crops. Only a few stakeholders strongly believe the delays in adoption were due to unpromising technology (25%), or low interest from farmers (27%).

Stakeholders identified key global issues that likely influence the adoption of modern biotechnology in their home countries (Figure 2). The stakeholders' responses indicated that most (80-84%) agree food security and climate change are critical drivers for adoption of modern biotechnology in Africa

The assessment also showed that top ranking causes of favorable attitudinal changes in the context of future acceptance of GM products by public would include, among others, scientific advancement (77%), better public awareness (74%), rising demand for food feed (67%), impacts of climate change (65%) as well as experiences from GM-adopting countries (65%) (Table 4).

Most stakeholders agreed the barriers for GM crop adoption come from socio-economic, ethical and socio-political concerns (76%), environmental concerns (62%), GM opponent pressure (61%) and perceived food safety and health concerns (57%). Participants identified these as likely reasons for slow adoption of GM crops and weak public acceptance (Table 5).

Analysis of 160 questions raised by 74 participants and recorded during the two courses at MSU indicated that close to 47% of stakeholder interest was about regulatory related issues (Figure 3). These questions were about risk assessment, biosafety approval processes, safety standards/protocols, authorization, product approval, legal issues, and safety considerations related to the environment, health, food and feed. Interest in technology development was the second highest issue raised (25%) by stakeholders who also expressed the need for more training support and capacity building in this area (Figure 3).

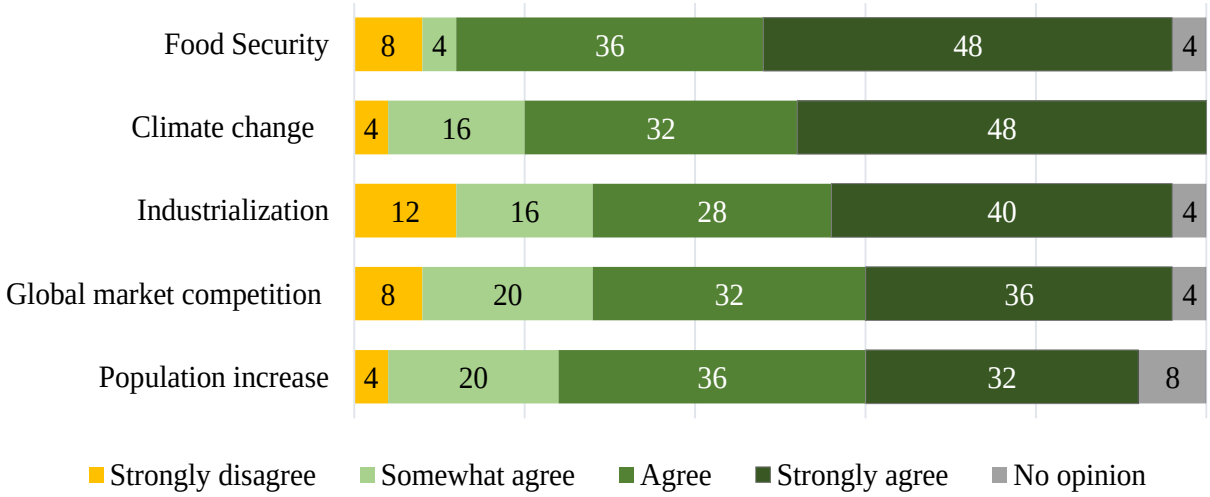

Figure 2. Global issues identified as likely drivers of biotechnology adoption (percentage responses).

Table 4. Stakeholders identification of factors favoring GM adoption.

Factor	Very likely (%)	Somewhat likely (%)	Not likely (%)	Do not know (%)
Promises of advances in science	77	9	5	9
Raising awareness of policy makers	74	13	4	9
Raising consumer demand	67	14	10	9
Prospect of climate change	65	26	0	9
Positive results from GM-adopting countries	65	13	13	9
Pressure from the scientific community	61	22	9	8
Rising role of media in favor of GM technology	57	17	17	9
Pressure from pro-GM advocates	40	30	13	17

DISCUSSION

Regulatory decisions

The surveys indicated that there is a need to build regulatory and technical capacity in Africa that will strengthen regulatory decision making and build public trust. Informed participants about GM products recognize the presence of delay in regulatory decision making and commercialization of biotech crops. More optimism is reflected in this assessment towards improved public attitude if awareness of stakeholders in GM technology is raised. Participants identified a number of drivers for adoption of biotechnology that include food security and climate change challenges. It has been suggested that linking regulatory decisions to national and United Nations Sustainable Development Goals provides an incentive for regulators to complete risk assessments and align decisions with national economic policy (Raybould, 2021). This focus on applying technology to drive development and sustainability would support regulatory decisions that could facilitate the adoption and commercialization of GM crops in Africa.

Appropriate technology

The key crop production and productivity challenges identified by these stakeholders relate to low adoption of improved technology due to poor access and availability of appropriate technologies that can respond to the farmers' specific production problems as well as poor agronomic conditions such as inadequate water, pests and diseases, poor soil fertility and climate change challenges. Improved genetics and best farming practices can reduce yield gaps and enhance productivity (Anthony and Feronni, 2011). Products derived through modern biotechnology such as GM and genome edited crops are relevant to meeting the needs of developing countries for food, feed and industrial applications (Anthony and

Table 5. Identified barriers to public acceptance of GM crops.

Causes of acceptance delay	Very likely (%)	Somewhat likely (%)	Not likely (%)	Do not know (%)
Fear of socio-economic, ethical and socio-political issues	76	10	10	5
Environmental risks concern	62	14	14	10
Pressure from GMO opponents	61	9	22	9
Concerns of food safety and potential health effects	57	14	19	10
Costly technology and approval processes	52	24	19	5
Inadequate farmers and public demand	52	29	10	10
Risk of market access loss	50	23	23	5
Fear of politicians losing votes	50	5	23	23
Do not perceive GM crops as beneficial	48	19	29	5
Too many actors are involved	33	33	29	5

Feronni, 2011; Gbashi et al., 2021). However, as the assessment showed, socio-economic, ethical or safety are key considerations along GM crops adoption that determines appropriateness of the technologies. Innovative GM crops developed with these perspectives in mind can solve production constraints and provide robust and sustainable solutions to increase food production and nutritional enhancement to help alleviate hunger and malnutrition (Cornish, 2018).

Commercialization and adoption in Africa

Most respondents of this survey replied that technology access and availability are the greatest barriers to wider adoption of GM crops. About two-third of the respondents observed an improving public attitude towards GM crops in recent years, but noted ongoing delays for approval and adoption of GM crops. Despite the availability of some appropriate technology, GM crop adoption in Africa has been slow (Alhassan et al., 2018; ISAAA, 2020). After the approval of Bt cotton in 1997 in South Africa, only Burkina Faso, Egypt, and Sudan commercialized this crop-trait combination 10-15 years later, between 2008 and 2012. The next approvals for Bt cotton in Africa were in Ethiopia, Eswatini and Nigeria in 2018, Kenya in 2019, and Malawi in 2020. Other early approvals for appropriate GM crops were for maize and soybean in 1997 and 2001 in South Africa. Nigeria approved Bt cowpea in 2019 and Bt-drought tolerant maize in 2021. Kenya approved virus resistant cassava in 2021. According to Aldemita et al. (2015), the speed of cultivation approval for GM crops globally from 1992-2014 shows remarkable contrast between Africa and the rest of the world. Between 1992 and 2003 there were 214 approvals in 16 countries globally whereas there were only 10 approvals in Africa all only in South Africa. In the next 14 years between 2004 and 2014, there were 419 approvals globally in 28 countries of which 12 approvals were only in 4 African countries: South Africa (9), Egypt (1), Burkina Faso (1) and Sudan (1).

It took more than 20 years for 7 of 46 SSA countries to adopt a range of improved GM crops. However, after GM crops are approved by national regulatory authorities, deployment of planting material to farmers has its own challenges.

Countries that adopted GM crops early have confirmed that appropriate technology can decrease production costs and increase food production (ISAAA, 2020). For instance, in Asia and Latin American countries, studies consistently confirm the adoption and progress of GMOs has been driven by economic value (Cornish, 2018; Brookes and Barfoot, 2014). In Brazil, GM soybean, maize and cotton varieties reduced farm production costs with an average farm income benefit of $34, $58 and $91 per hectare, respectively (Cornish, 2018). Participants in this survey confirmed that although farmers show interest to grow GM crops, issues of access to new technology, seed cost and market linkage are potential barriers for farmers to adopt GM crops. Piñeiro et al. (2020) similarly indicated that for smallholder farmers, sustainable access and affordability of planting material are important success factors in adoption of GM crops. Therefore, product commercialization requires careful planning from the start to avoid delays and provide sustainable access to high quality planting material for farmers. Discussions on commercialization start early in product development to confirm acceptance and evaluate marketing plans for the final product. When approved GM crops are transferred for adoption in other countries, the commercialization plans need to be adapted and developed considering specific local circumstances to ensure successful dissemination. Potential market value and local desire for the traits should be the driving force for technology transfer. It can be seen from the above assessment that technology access constraints are diverse

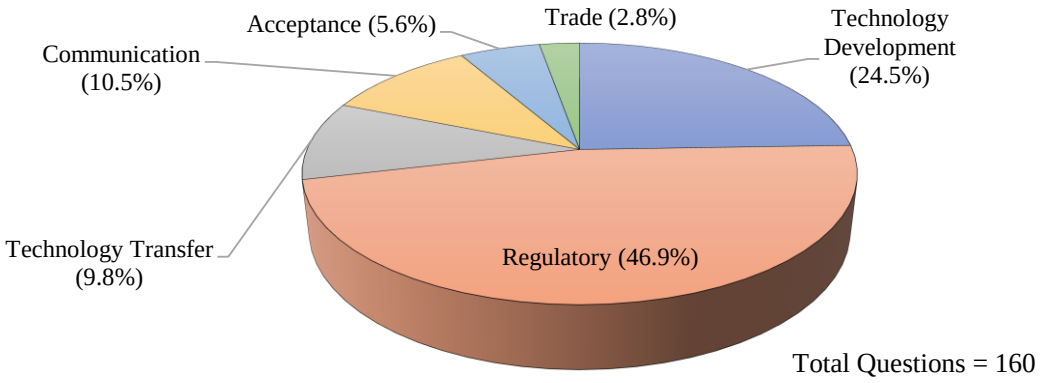

Figure 3. Stakeholder questions categorized by thematic area.

and can effectively hinder the wide adoption of GM crops. In Africa, the presence of functional seed and extension systems, adequate stakeholder awareness, effective technology demonstration, financial schemes to support smallholder farmers and reliable marketing services all require shared responsibility and coordination between developers and government services.

Seed systems in Africa

Dissemination and adoption of improved new varieties in Africa is relatively slow due also to a weak private seed sector and dominant informal seed systems. According to Akinbo et al. (2021), in SSA the informal seed sector accounts for about 80% of the seeds planted and, in most countries, public sector seed programs play a major role in supplying quality seeds for registered and released varieties. A thriving seed system requires a growing demand for seed and well-functioning markets for seed and grain, a robust innovation system for the crop improvement, and an effective regulatory system to sustain an innovative and competitive market (Spielman and Kennedy, 2016). In developing countries, it is hard to find these components fully in place. The case of maize in Asia is a good example of how multinational seed companies with strong R&D programs and product lines played a central role in those markets, operating independently or in joint ventures with domestic seed companies (Spielman and Kennedy, 2016).

The private sector leadership in Asia's maize seed market did not entirely replace the need for public research. In fact, in Thailand, combination of policy reforms and a strong public-sector maize development program in the 1970s transitioned the country into a hub for private R&D investment. The key challenge with the public system, however, is to remain competitive due to limitations in scientific capacity, funding trends, top-heavy organizational structures, and poor research and technology promotion incentives (Schreinemachers et al., 2021; Spielman and Kennedy, 2016).

Commercialization of hybrid seed, open pollinated seed and vegetative planting material has well documented challenges in Africa (Schreinemachers et al., 2021). These authors confirm that seed regulations are easing in a number of African countries to encourage investment and establishment of private seed companies. They encourage policy makers to create favorable commercial and regulatory environments for seed companies; incentives to strengthening investment and capacity in seed research and development; provision of extension programs to encourage adoption of improved farming methods; and infrastructure for access to markets for farm produce. Mbabazi et al. (2020) noted that lack of technical capacity and infrastructure to implement seed delivery systems leads to delayed access to and adoption of improved planting material in Africa.

Technology delivery: Institutional and stakeholders coordination and partnership

Participants of this survey indicated that social, economic and political challenges are critical concerns that affect wider acceptance of GM crops in their countries. Leading up to commercialization, effective demonstration and awareness creation among the extension agents and smallholder farmers determines the adoption of an approved GM crop. Many African farmers do not routinely purchase improved seeds despite the demonstrated potential of these technologies to improve productivity and success. It shows the transfer of GM crop technology to Africa will benefit from strong public-private partnerships that ensure a reliable source of good planting material for farmers and all the support the public sector can give them to bring a good crop to market. This is illustrated in a recent assessment report by WorldTAP Program, MSU (2021) on the challenges of bridging the

Bt cotton seed access gap and ensuring a sustainable supply of seeds to smallholder farmers. Others have also highlighted the role successful public-private partnerships can play in effectively commercializing an approved GM crop (Mbabazi et al., 2020).

Lesson learnt and way forward

Commercialization of GM products is a multifaceted, long-term and expensive undertaking. It takes 10-15 years to commercialize a GM crop with high costs. Private sector has played a dominant role in commercialization of GM crops to date. Public-private sector partnerships are critical to facilitate commercialization of GM crops in developing countries. Although concerted efforts are ongoing to build scientific and regulatory capacity and to deploy appropriate GM crops from the international community, there continue to be delays in regulatory approvals for general release and commercialization of GM crops in Africa.

Lack of political will, safety concerns, fear of change and negative public perception have contributed to delays in adoption. However, during the past three years, there has been a wind of change in Africa with several countries taking positive steps and making favorable regulatory decisions that facilitate the commercialization of GM crops. In this respect, Raybould (2021) reports that many countries have demonstrated political will to assess and harness GM crops. Linking national regulatory decisions on GM crops to national policy goals, such as achieving the United Nations Sustainable Development Goals, will help to clarify which products benefit the local society, the environment, and economic growth. These linkages will reinforce that science is helping to achieve policy aims, not driving its own agenda.

Biosafety regulatory decisions are just one of the many steps involved in the commercialization process that will put GM seeds in the hands of smallholder farmers. Vibrant seed systems, local seed production capacity, effective extension services, reliable financial and marketing services as well as product stewardship strategies are needed to deploy and sustain GM crops for smallholder farmers. Unless these issues are addressed, smallholder farmers in Africa will not benefit from GM crops.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

REFERENCES

Akinbo O, Obukosia S, Ouedraogo J, Sinebo W, Savadogo M, Timpo S, Mbabazi R, Maredia K, Makinde D, Ambali A (2021). Commercial release of genetically modified crops in Africa: interface between biosafety regulatory systems and varietal release systems. Frontiers in Plant Science 12:605937.

Aldemita RR, Reaño IME, Solis RO, Hautea RA (2015). Trends in global approvals of biotech crops (1992–2014). GM Crops and Food 6(3):150-166.

Alhassan SW, Anthony VM, Rudelsheim P (2018). Implementation of stewardship in African agricultural public research and development institutions. Journal of Agricultural Biotechnology and Sustainable Development 10(5):97-103.

Anthony VM, Ferroni M (2011). Agricultural biotechnology and smallholder farmers in developing countries. Current Opinion in Biotechnology 23(2):278-285. doi:10.1016/ j.copbio.2011.11.020

Azadi H, Samiee A , Mahmoudi H, Jouzi Z , Khachak PR, De Maeyer P, Witlox F (2015). Genetically modified crops and small-scale farmers: main opportunities and challenges. Critical Reviews in Biotechnology 36(3):434-446. doi:10.3109/07388551.2014.990413.

Bayer Crop Science (2020). GMO & Biotechnology Unlocking Hidden Potential. https://www.cropscience.bayer.com/ innovations/seeds-traits/gmo-biotechnology.

Bennett R, Morse S, Ismael Y (2006). The economic impact of genetically modified cotton on South African smallholders: Yield, profit and health effects. The Journal of Development Studies 42(4):662-677.

Brookes G, Barfoot P (2014). Economic impact of GM crops. GM Crops and Food 5(1):65-75, DOI: 10.4161/gmcr.28098

Cornish L (2018). What are the political drivers for GMOs in developing countries? devex News. Inside Development. The GMO debate, 2021

EUROPEAN COMMISSION (EU) (2010). A decade of EU-funded GMO research (2001-2010). https://www.researchgate.net/publication/233770770. ISBN: 978-92-79-16344-9.

Excellence through Stewardship (ETS) (2017). Guide for Stewardship of Biotechnology Derived Plant products. https://www.excellencethroughstewardship.org/ets-guides

Gbashi S, Adebo O, Adebiyi JA, Targuma S, Tebele S, Areo OM, Olopade B, Odukoya JO, Njobeh P (2021). Food safety, food security and genetically modified organisms in Africa: a current perspective. Biotechnology and Genetic Engineering Reviews 37(1):30-63. doi: 10.1080/02648725.2021.1940735. ISAAA (2018). Brief 54: Global status of commercialized biotech/GM crops

ISAAA (2020). Brief 55: Global status of commercialized biotech/GM crops.

ISAAA Briefs 54 (2018). Global Status of Commercialized Biotech/GM Crops in 2018. https://www.isaaa.org/resources/publications/briefs/54/

ISAAA Crop Biotech Update (2010). EC Report on "A Decade of EU-funded GMO Research" Describes "Tailored" Bioenergy Crop Research Project. http://ec.europa.eu/research/biosociety/pdf/a_decade_of_eu-funded_gmo_research.pdf

Kouser S, Qaim M (2011). Impact of Bt cotton on pesticide poisoning in smallholder agriculture: a panel data analysis. Ecological Economics 70(11):2105-2113. Doi: 10.1016/j.ecolecon.2011.06.008.

Mbabazi R, Dissanayake HG, Guenthner J, Maredia K (2016). Perception of International Stakeholders on Genetically Modified Organisms (GMOs). Asian Biotechnology and Development Review 18(2):51-57.

Mbabazi R, Koch M, Maredia K, Guenthner J (2020). Crop Biotechnology and Product Stewardship. Biotechnology in Agriculture and the Food Chain 12(1):106-114

NASAC (2015). Harnessing Modern Agricultural Biotechnology for Africa's Economic Development. Recommendations to Policymakers. Network of African Science Academies (NASAC). Dezine Creationz Ltd. Publishers.

Noisette C (2021). World and EU- Decrease in the area of transgenic GMOs in 2019. https://www.infogm.org/7145-world-and-eu-decrease-area-transgenic-gmos-2019?lang=fr

Oloo B, Maredia K, Mbabazi R (2020). Advancing adoption of

genetically modified crops as food and feed in Africa: The case of Kenya. African Journal of Biotechnology 19(10):694-701. DOI: 10.5897/AJB2020.17159

Piñeiro V, Arias J, Dürr J, Elverdin P, Ibáñez AM, Kinengyere A, Opazo CM, Owoo N, Page JR, Prager SD, Torero M (2020). A scoping review on incentives for adoption of sustainable agricultural practices and their outcomes. Nature Sustainability 3(10):809-820. https://doi.org/10.1038/s41893-020-00617-y

Raybould A (2021). Improving the politics of biotechnological innovations in food security and other sustainable development goals. Transgenic Research 30(5):613-618. doi: 10.1007/s11248-021-00277-4.

Schreinemachers P, Howard J, Turner M, Groot SN, Bhupen D, Mwadzingeni L, Chagomoka T, Ngugi M, Afari-Sefa V, Hanson P, Wopereis MCS (2021). Africa's evolving vegetable seed sector: status, policy options and lessons from Asia. Food Security 13(3):511-523. https://doi.org/10.1007/s12571-021-01146-y

Silas DO, Maredia K, Kedisso EG, Guenther J, Jeremy O (2022). Value-chain of cotton industry in Kenya with focus on product stewardship for timely provision of certified quality seeds to farmers. African Journal of Biotechnology (*In press*).

Spielman DJ, Kennedy A (2016). Towards better metrics and policymaking for seed system development: Insights from Asia's seed industry. Agricultural Systems 147:111-122

Turnbull C, Lillemo M, Hvoslef-Eide TAK (2021). Global Regulation of Genetically Modified Crops Amid the Gene Edited Crop Boom – A Review. Frontiers in Plant Science 12:630396. doi: 10.3389/fpls.2021.630396

USDA Foreign Agricultural Services (2020). Agricultural Biotechnology Annual Report: Ethiopia. Report Number: ET2019-0010.

Waithaka M, Belay B, Kyotalimye M, Karembu M (2015). Progress and challenges for implementation of the Common Market for Eastern and Southern Africa policy on biotechnology and biosafety. Frontiers in Bioengineering and Biotechnology 3:109. doi:10.3389/fbioe.2015.00109.

Vol. 21(5), pp.198-207, May 2022
DOI: 10.5897/AJB2021.17425
Article Number: 45A319069078
ISSN: 1684-5315

http://www.academicjournals.org/AJB

ACADEMIC JOURNALS
expand your knowledge

African Journal of Biotechnology

Full Length Research Paper

Preliminary verification of the adoption status of some yam (*Dioscorea rotundata* and *Dioscorea alata*) varieties in Nigeria using microsatellites markers

Nwogha J. S.[1*], J. E. Obidiegwu[1], R. N Okereke[1], R. Bhattacharjee[2] and H. O. Oselebe[3]

[1]National Root Crops Research Institute, Umudike, Umuahia, Abia State, Nigeria.
[2]International institute for Tropical Agriculture, (IITA), Ibadan, Nigeria.
[3]Department of Crop Production and Landscape Management, Faculty of Agriculture & Natural Resource Management, Ebonyi State University, Abakaliki, Nigeria.

Received 29 October, 2021; Accepted 17 December, 2021

The persistent low yield and farmers' preference of their traditional yam varieties over the improved varieties necessitated this study to verify the adoption status of the released varieties in Nigeria. A total of 48 accessions of white yam (*Dioscorea rotundata*) were sampled from six states of Ebonyi, Enugu, Benue, Kogi, Nassarawa and Oyo within Nigeria yam-belt and were genotyped for relatedness to four released varieties from the National Root Crops Research Institute (NRCRI), Umudike yam breeding programme, while 14 accessions of water yam (*D. alata*) were sampled from four states of Benue, Kogi, Nassarawa and Oyo and were also genotyped for relatedness to three released varieties from International Institute for Tropical Agriculture (IITA), Ibadan. A total of 29 alleles were found in 5 sets of primers analyzed for 52 *D. rotundata* accessions and the number of alleles ranged from 5 (Dald08, SSR 51 and YM 34) to 8 (Dab2E07) with an average of 5.8 per locus. The observed heterozygosity ranged from 0.19 (YM34) to 0.77 (YM30). A total gene diversity of 0.63 according to Nei (1978) genetic distance coefficients was observed among the 52 *D. rotundata* accessions. Similarly, a total of 37 alleles were observed when 17 *D. alata* accessions were analysed with the 7 selected sets of primers. An average of 5.29 alleles was observed per locus. The observed heterozygosity varied from 0.47 (Dab2D06) to 0.82 (YM34). A total gene diversity of 0.58 was observed among 17 *D. alata* accessions according to Nei' genetic distance coefficients. Cluster analysis showed that the *D. rotundata* accessions were classified into 8 clusters. While, 17 accessions of *D. alata* were classified into 4 clusters. There were relationships between some released varieties and farmers accessions and also among the farmers' accessions from different locations, indicating that farmers might have given a preferred local name to the released varieties.

Key words: Verification, adoption, status, yam, microsatellites-markers.

INTRODUCTION

Yam (*Dioscorea* species), a vegetatively propagated crop cultivated for its underground edible tubers, is a crucial food and income source for millions of Nigerians. Nigeria alone accounts for 65% of global yam production. About 48 million tons of yams are produced in Nigeria from 5.9 million hectares of land (FAO, 2018). Yam ranks as an important source of dietary calories. Between 2006 and 2010, 300 million people derived an average of more than 200 kilocalories per person per day from yam (Nweke et al., 2013). Hence, yam is important for food security and income generation for at least 60 million people (that is, domestic retail price of $0.49 per kg for

the 48 million tons produced in Nigeria). A typical yam farmer in Nigeria has an average of 2.38 ha of farmland, of which 1.53 ha (64%) is dedicated to yam production. Yam is also integral to the socio-cultural life of the people (Obidiegwu and Akpabio, 2017).

Yams belong to the monocotyledonous *Dioscorea* genus. This genus has about 613 species and about 10 of *Dioscorea* spp. have been domesticated (Ayensu and Coursey, 1972). *Dioscorea rotundata* (white yam) and *Dioscorea alata* (water yam) are two dominant species of economic importance in Nigeria. Studies on efficiency-equity trade-offs and poverty-based priority setting have together demonstrated the possibility of directing greater benefits to the poor through yam improvement (Alene and Hassan, 2006; Alene and Manyong, 2007). If improved varieties and sustainable technology were used by farmers, at least a 30% yield increase will be actualised within the same area of production annually (YIIFSWA Yamnomics Factsheet, 2016). While population growth is on the increase, yam productivity per hectare is declining. Since 2000, the rate of annual increase in yam production has been decelerating compared to earlier dramatic increases associated with area expansion into the savannah. It has been predicted that this decrease could be catastrophic unless expedited steps are taken. The decline in productive potential is attributed to a combination of factors mostly associated with the intensification of cultivation due to shortened fallow periods, deteriorating soil fertility, poor seed quality, and inadequate yield potential of popular yam varieties and landraces. These obstacles reduce the total food supply in Nigeria, undermine value chain stakeholder's ability to generate sustainable incomes, and disproportionately impact rural women.

In a bid to meet the aforementioned targets, yam improvement strategy has been deployed since 1980s. National Root Crops Research Institute (NRCRI) Umudike, Nigeria and International Institute for Tropical Agriculture (IITA) have been at the forefront of developing market-oriented varieties with the first release in year 2001. The *D. rotundata* is believed to have originated from West Africa (Maass et al., 2007; Scarcelli et al., 2019; Yu et al., 2020). The domestication process has led to the evolution of numerous landraces across Nigeria. These farmers preferred landraces remain the benchmark for varietal improvement. *D. alata* is believed to have originated from South East Asia (Nabeshima et al., 2020) and subsequently introduced to other parts of the world including West Africa. Some of these introduced varieties have been cultivated over years through selection process. These landraces have been inherent in them. Due to the cultural ties of the local yam farming communities, it is envisaged that most of the released yam varieties end up being converted to the locally preferred names by farmers. This has created bottlenecks in varietal tracking and identification based on the clonal attributes of yam. Till date there is no evidenced based study that tries to validate the status of yam adoption in Nigeria, Morphological markers are not reliable in identifying yam varieties, and molecular markers like SSRs have been revealed to be reliable alternative (Obidiegwu et al., 2009b, c; Siqueira et al., 2011; Silva et al., 2016). This preliminary study was conducted to verify the adoption status of seven (7) released varieties including *D. rotundata* and *D. alata* in Nigeria using Simple Sequence Repeats (SSRs).

MATERIALS AND METHODS

Plant

For this study, six (6) states were selected based on their active participation on National Coordinated Research programs and on farm verification trials prior to the release of improved varieties. These states include Ebonyi, Enugu, Benue, Nasarawa, Kogi and Oyo known for being major yam producing regions in Nigeria (Figure 1). The particular sample locations and their coordinates are presented in Table (1).

Forty-eight (48) *D. rotundata* and fourteen (14) *D. alata* leaves were sampled from sixty-two individual plants. The seven released improved varieties (4 *D. rotundata* and 3 *D. alata*) to be identified were sampled from the yam germplasm managed by the 2International institute for Tropical Agriculture (IITA) and National Root Crops Research Institute (NRCRI) yam breeding programmes (Table 2). All samples were collected into conical filter paper and store in silica gels at room temperature to dry.

DNA extraction

Silica gel dried leaf samples were transported to the Bioscience Laboratory, IITA, Ibadan, Nigeria. DNA was isolated from 100 mg of dried yam leaf samples using a modified CTAB extraction method as described by Sharma et al. (2008). The DNA extracts were eluted in 100 µl sterile TE buffer and the quality and concentration were assessed by gel electrophoresis using 1% agarose with known concentrations of undigested lambda DNA (Sigma, St Louis, MO, USA). The extracts were further quantified using a Nanodrop spectrophotometer and stored at -20°C for genotyping. Prior to PCR analysis, the samples were standardized to 25 ng/µl.

DNA quality and molecular concentration

All 52 samples of *D. rotundata* and 17 samples of *D. alata* produced high molecular weight DNA with very good quality. The average concentration of DNA extracted was 2707.4 ng/µl, ranging from 1277.8 to 4317.3 ng/µl. Purity of the DNA extracts was on the scale of A260/280 and an average of 1.88, ranging from 1.78 to 1.95.

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

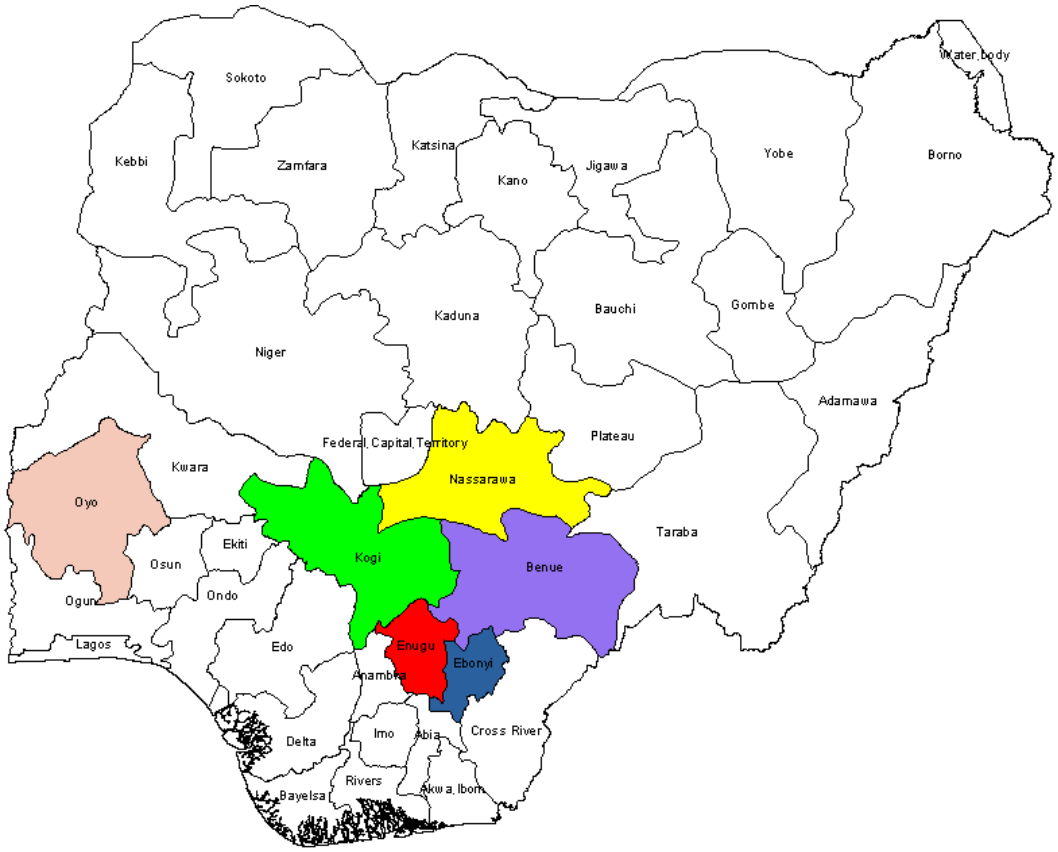

Figure 1. Nigerian administrative map showing the geographic location of the states where samples were collected.
Source: Author

PCR genotyping

Twenty-three SSR primer pairs developed from various yam species were selected and used to analyse the samples according to Tamiru et al. (2015) and Tostain et al. (2007). The primer pairs were tested on the released varieties and five (5) were chosen for *D. rotundata* and seven (7) for *D. alata* based on the capacity to reveal polymorphism and reproducibility. PCR reactions were conducted on a thermocycler (Applied Biosytems Veriti 96 well plates) in 10 µl volumes containing 1 µl of template DNA, 100 µM each of dNTP, 2.5 mM $MgCl_2$, 0.5 µM, 10X reaction buffer and 2 units of Taq DNA polymerase. Depending on the loci used, different touch down PCR cycle program was used. The PCR amplicons were electrophoresed on 2% agarose gel in 0.5 TBE buffer along with a DNA molecular size marker. Gels were photographed using a gel documentation system.

Gel scoring and fragment analysis

PCR amplicons (2 µL) were mixed with loading dye and subjected to further electrophoresis in 6% polyacrylamide gels at 100 V for 30 min. The resolved and unambiguous DNA bands generated from the separation of the PCR products were counted by starting from the top to the bottom of the lanes and were also scored as presence (1) and absence (0) of bands. The binary data generated was analysed using Gstudio (Rodney, J.D.) R package, to determine genetic diversity parameters such as allele numbers per locus (A), the effective allele numbers per locus (Ae), polymorphic information content (PIC), observed (Ho), and expected (He) heterozygosity, respectively, genetic distant, as well as cluster analysis.

RESULTS

Genetic diversity across yam accessions

A total of 29 alleles were found in 5 sets of primers analyzed for 52 *D. rotundata* accessions. The number of alleles ranged from 5 (Dald08, SSR 51 and YM 34) to 8 (Dab2E07) with an average of 5.8 per locus (Table 3). The observed heterozygosity (Ho) ranged from 0.19 (YM34) to 0.77 (YM30). Genetic distance coefficients were observed among the 52 *D. rotundata* accessions. The discriminative power of each SSR primer was assessed using the polymorphic information content (PIC). PIC values ranged from 0.58 (Ym34) to 0.73 (Ym30) with an average of 0.64. Similarly, a total of 37 alleles were observed when 17 *D. alata* accessions were analysed with the 7 selected sets of primers. An average of 5.29 alleles was observed per locus (Table 4). The observed heterozygosity varied from 0.47 (Dab2D06) to

Table 1. List of sampled accessions, sample states and sample site physical coordinates.

Sample state	Sample local name	Sample location coordinates
D. rotundata		
Ebonyi	Agric-3_EB	06°20'13.3N" 008° 11'47.3E"
Ebonyi	Agric-4_EB	06°20'13.3N" 008° 11'47.3E"
Ebonyi	Agric-5_EB	06°20'13.3N" 008° 11'47.3E"
Enugu	Agric-2_EN	06°14'50.9N" 007°25'50.0E"
Enugu	Agric-1_EN	06°14'50.9N" 007°25'50.0E"
Kogi	Unknown-1_KG	07°26'23.9N" 007°35'27.1E"
Kogi	Unknown-2 _KG	07°26'23.9N" 007°35'27.1E"
Kogi	Unknown-3 _KG	07°27'20.1N" 001°37'17.8E"
Kogi	Unknown-4 _KG	07°27'20.1N" 001°37'17.8E"
Kogi	Unknown-5 _KG	07°27'20.1N" 001°37'17.8E"
Kogi	Unknown-6 _KG	07°27'20.1N" 001°37'17.8E"
Kogi	Unknown-7 _KG	07°27'20.1N" 001°37'17.8E"
Oyo	Lasmi-2	09°05'52.3N" 003°49'38.3E"
Oyo	Lasiri-3	09°05'52.3N" 003°49'38.3E"
Oyo	Ojuiyawo	09°05'52.3N" 003°49'38.3E"
Oyo	Ajidawa	09°07'03.7N" 003°53'18.1E"
Oyo	Zaria	09°07'03.7N" 003°53'18.1E"
Oyo	Sofinni	09°07'03.7N" 003°53'18.1E"
Oyo	Lasiri-4	09°07'03.7N" 003°53'18.1E"
Oyo	Agbaowobe	09°07'03.7N" 003°53'18.1E"
Oyo	Ndu	08°47'29.0N" 003°48'47.2E"
Oyo	Ojuiyawo-3	08°47'25.5N" 003°48'43.4E"
Oyo	IITA 1	08°47'38.5N" 003°48'47.3E"
Oyo	Ketuketu	08°47'23.5N" 003°48'43.4E"
Oyo	Talaaba	08°47'38.5N" 003°48'47.3E"
Oyo	Lasiri-1	08°47'38.5N" 003°48'47.3E"
Oyo	Lasiri-2	08°47'38.5N" 003°48'47.3E"
Oyo	Yalanba	08°49'46.4N" 003°46'59.4E"
Oyo	Ofegi	08°49'46.4N" 003°46'59.4E"
Oyo	Ehuru	08°49'46.4N" 003°46'59.4E"
Oyo	Lasiri	08°49'46.4N" 003°46'59.4E"
Oyo	Ojuiyawo-1	08°49'46.4N" 003°46'59.4E"
Nasarawa	Dandiopu	08°22'55.1N" 008°36.45.0E"
Nasarawa	Pepa	08°22'55.1N" 008°36.45.0E"
Nasarawa	Okah	08°22'55.1N" 008°36.45.0E"
Nasarawa	Adaka	08°22'55.1N" 008°36.45.0E"
Nasarawa	Aloshi	08°22'55.1N" 008°36.45.0E"
Nasarawa	Hembakwesi (NAS)	08°22'55.1N" 008°36.45.0E"
Nasarawa	Aloshi	08°29'33.8N" 008°34'34.2E"
Nasarawa	Ame	08°29'33.8N" 008°34'34.2E"
Nasarawa	Pepa	08°29'33.8N" 008°34'34.2E"
Benue	Hembakwesi (BEN)	07°42'51.0N" 008°41'06.2E"
Benue	Unknown-1_BEN	07°42'51.0N" 008°41'06.2E"
Benue	Unknown-2_BEN	07°42'59.1N" 008°41'02.9E"
Benue	Tokula_BEN	07°42'59.1N" 008°41'02.9E"
Benue	Tokula_BEN-1	07°42'59.1N" 008°41'02.9E"
Benue	Unknown -3_BEN	07°42'51.0N" 008°41'06.2E"
Benue	Unknown-4 _BEN	07°42'51.0N" 008°41'06.2E"
D. alata		
Oyo	Agric-2	08°47'38.5N" 003°48'47.3E"

Table 1. Contd

Oyo	Agric-3	08°49'46.4N" 003°46'59.4E"
Oyo	Ehura	08°47'23.5N" 003°48'43.4E"
Oyo	Oharan	08°49'46.4N" 003°46'59.4E"
Oyo	Agric	09°05'52.3N" 003°49'38.3E"
Oyo	Boko	08°47'38.5N" 003°48'47.3E"
Oyo	Agric-1	08°47'58.5N" 003°48'47.3E"
Kogi	Local-1	07°27'20.1N" 007°37'17.8E"
Kogi	Local-2	07°27'20.1N" 007°37'17.8E"
Nassarawa	Shakata	08°22'55.1N" 008°36'45.0E"
Benue	Tokula-1	07°42'59.1N" 008°41'02.9E"
Benue	Tokula-2	07°42'59.1N" 008°41'02.9E"
Benue	Tokula-3	07°42'59.1N" 008°41'02.9E"
Benue	Local-3	07°42'51.0N" 008°41'06.2E"

0.82 (YM34). A total gene diversity of 0.58 was observed among 17 *D. alata* accessions according to Nei' genetic distance coefficients. All seven primer pairs produced amplicons with varying levels of polymorphism revealing an average of 69.81% polymorphism. The polymorphic information content ranged from 0.42 (Dab20C5) to 0.69 (Dab2E07) with an average of 0.66.

Genetic relatedness among accessions

The genetic distance for the microsatellite data using 52 *D. rotundata* and 17 *D. alata* accessions were constructed based on Nei (1978) and the relationships between accessions were depicted on dendogram graphs. The *D. rotundata* accessions were classified into 8 clusters (Figure 2), with released variety TDr89/02461 in cluster 5 and other released varieties TDr 95/19158, TDr 89/02665 and TDr 89/02677 in cluster 8. Cluster 5 is further classified into 2 subgroups, with TDr 89/02461 in the sub-cluster with Pepa sampled from Nasarawa and Lasri-4, sampled from Oyo. Cluster 8 is made up of 3 subgroups. In group1 TDr 95/19158 is clustered with Ajidawa from Oyo State, while TDr 89/02665 clustered with Lasiri and Lasiri-2 sampled also from Oyo State, as well as unnamed variety (Unknown-1_KG) sampled from Kogi State as seen in group 2. Group 3 showed that TDr 89/02677 is clustered with Lasmi-2 sampled from Oyo, with unnamed varieties sampled from Kogi (Unknown-4_KG) and Benue (Unknown-2_BEN) and also Tokula-1 from Benue as well. Figure 3 shows that 17 accessions of *D. alata* were classified into 4 clusters. Two of the released varieties (TDa 00/00194 and TDa 98/01176) are in cluster 3. Two accessions, local-3 and Tokula-2 sampled in Benue from two different farmers also show close relationship with each other and cluster also with released varieties (TDa00/00194 and TDa98/01176) as seen in cluster 3. Released variety TDa98/01168 as shown in cluster 4 is clustered with accessions (Agric-2 and Agric-1) from Oyo, (Tokula-1) from Benue and (Local-2) from Kogi.

DISCUSSION

The genetic diversity

D. rotundata

The present study provides a baseline for evaluating the adoption rate of some released varieties. On the average, 5.8 alleles per locus were detected for *D. rotundata*. Genetic diversity of 0.63 was observed among the *D. rotundata* accessions. These results demonstrate a sufficient genetic polymorphism in accessions of both species sampled from farmers' field and improved varieties in research institutes' managed breeding programmes. The genetic diversity among the accessions provides insight on the range of genetic base of cultivars that were used in the study. Gene diversity of 0.63 observed among D*. rotundata* accessions indicates that samples utilized in this study constitute a substantive proportion of Nigerian *D. rotundata* diversity, when compared with 0.677 gene diversity reported by Obidiegwu et al. (2009b) after sampling the whole country. This result also suggests a wide range of genetic diversity of *D. rotundata* accessions that were used in this study and equally reaffirm the inference that Nigeria is a centre of its diversity. It has been reported that the high genetic diversity among yam accessions might be due to the crop being vegetatively propagated which usually maintains high heterozygosity (Siqueira et al., 2011). Similarly, Obidiegwu et al. (2009b) stated that the high genetic diversity of *D. rotundata* accessions is due to its dioecious nature and thus, spontaneous hybridization must have contributed to the ancestry of some of the accessions. Meanwhile, the selection of somatic mutants by farmers might also be a source of genetic variability in

Table 2. Released *D. rotundata* and *D. alata* varieties, used, with year of release and improved qualities.

Released variety	Outstanding characteristics	Year of release
D. rotundata		
TDr 89/02677	Stable yield, very good cooking and pounding qualities, cream tuber parenchyma, 25% tuber dry matter content.	2001
TDr 89/02461	Stable yield, very good as cooking and pounding qualities, cream parenchyma, 26.7% tuber dry matter	2001
TDr 89/02665	Stable yield, very good cooking and pounding qualities, cream non-oxidizing parenchyma, 35.3% tuber dry matter.	2003
TDr 95/19158	High yielding, pests and diseases tolerant, good for pounded yam, frying and boiling (29.4 t/ha)	2008
D. alata		
TDa 98/01168	High yielding, pests and diseases tolerant, good for pounded yam, frying and boiling (24-28 t/ha)	2008
TDa 00/00194	High yielding, pests and diseases tolerant, very good for yam, fufu, frying and boiling (37.5 t/ha)	2009
TDa 98/01176	High yielding, pests and diseases tolerant, very good for pounded yam, frying and boiling, suitable for both rainy and dry seasons yam production (26-30 t/ha)	2008

their plant improvement practices. Toasten et al. (2007) attributed a similar genetic diversity of 0.56 among *D. rotundata* in Benin Republic to genetic mutation as result of adaptation mechanism to marginal environment such as very poor soils, flood and long periods of drought as well as farmers' strong selection.

D. alata

An average of 5.3 alleles per locus and genetic diversity of 0.58 were observed among *D. alata* accession. The relative low diversity among the accessions of *D. alata* compared to *D. rotundata* suggests a narrower genetic base of *D. alata* accessions used. This could be attributed to small number of states (four) within Nigeria's yambelt. Therefore, these accessions might not be a good representative of Nigeria's *D. alata* germplasm. This assertion is supported by the findings of Obidiegwu et al. (2009c) who reported genetic diversity of 0.669 among *D. alata* accessions in Nigeria indicative of a wide range of its genetic base in Nigeria. Although, sampling took place only in four states of the thirty-six in Nigeria, those states ((Oyo, Benue, Kogi and Nassarawa) are the hotspots of yam production in Nigeria and thus were expected to be a good representative of *D. alata* germplasm in the country.

PIC values ranged from 0.58 to 0.73, with an average of 0.64 for *D. rotundata* accessions. While PIC values ranging from 0.42 to 0.69 with an average of 0.66 were observed in *D. alata* accessions. PIC refers to the value of a marker for detecting polymorphism within a population, d**e**pending on the number of observable alleles and the distribution of their frequency. It has been proven to be a general measure of how informative a marker is (Siqueira et al., 2011). PIC values in this study demonstrate that the SSRs used on average presented a high level of information and were sufficiently discriminatory. Similarly, PIC values have been reported in previous studies by Tostain et al., 2007; Obidiegwu et al., 2009b, c; Siqueira et al., 2011).

Genetic relatedness among accessions

In this study, the adoption status of the released varieties were evaluated using the genetic distance of microsatellite data from 52 *D. rotundata* and 17 *D. alata* accessions to construct cluster dendrograms based on Nei (1978). The relationships between released and farmers' accessions are also shown on dendrogram graphs. *D. rotundata* accessions were distributed into 8 clusters (Figure 2), with released varieties: TDr 95/19158, TDr 89/02665 and TDr 89/02677 in cluster 8, indicating that these varieties might be progenies of common genetic pedigree. In addition, they also share cluster 8 with many other farmers' accessions, indicating diverse genetic relationships with farmers' accessions from various locations. For instance, TDr89/02677 is closely related to Lasiri from Oyo, Unknown-4_KG from Kogi, Tokula_BEN-2 and Unknown-2_BEN. The nature of the linkage indicates that the farmers' accessions shared genetic background with the released variety TDr89/02677 or can even be its mutants but with little genetic variations due to adaptation to different environments as earlier adduced by Tostain et al. (2007). Similarly, released variety TDr89/02665 was closely related to Lasiri-2 from Oyo or can be the same as Lasiri-2, but with farmers' name and not breeders' code. While Unknown-1_KG from

Table 3. Characteristics of SSR markers used in analysis of 52 *D. rotundata* accessions.

Locus	Sequence	A	Ae	Hobs	PIC
Dald08	AATGCTTCGTAATCCAAC – F CTATAAGGAATTGGTGC - R	5	2.61	0.71	0.62
YM 30	CCACAACTAAAAACACATGGAC - F GTGGTAGGGTGTGTAGCTTCTT - R	6	3.63	0.77	0.73
Dab2E07	TTGAACCTTGACTTTGGT – F GAGTTCCTGTCCTTGGT -R	8	2.94	0.46	0.66
SSR 51	GAATACATATGGTGCATTCGAG - F GCTGCTTACAACTGACAAAGTC - R	5	2.58	0.29	0.61
YM 34	GGTAATAGAGGGCAAAGTGGC - F AGACCTCCTACCATGCTCAAG – R	5	2.40	0.19	0.58
Average		5.8	2.83	0.48	0.64

Number of alleles per locus (A), observed heterozygosity (Ho), effective alleles (Ae) and polymorphic information content (PIC).
Source: Author's computation.

Table 4. Characteristics of SSR markers used in analysis of 17 *D. alata* accessions.

Primers	5' to 3' Primer sequence	*A*	Ae	Hobs	PIC
Dab20C5	CCCATGCTTGTAGTTGT -F TGCTCACCTCTTTACTTG -R	3	1.74	0.53	0.42
D 83	TCGGAATTCAACTGTGATGGC -F AGCACACCATTCACACATAGG -R	6	2.56	0.59	0.61
D 100	GTGTGTGGATGGAGTTTCAAT -F GAATACCCCCAACAGATGTAAT -R	5	2.32	0.77	0.57
Dab2E07	TTGAACCTTGACTTTGGT -F GAGTTCCTGTCCTTGGT -R	6	3.18	0.65	0.69
Ym 34	GGTAATAGAGGGCAAAGTGGC -F AGACCTCCTACCATGCTCAAG -R	5	2.44	0.82	0.59
Ym 51	GAATACATATGGTGCATTCGAG -F GCTGCTTACAACTGACAAAGTC -R	7	2.98	0.57	0.66
Dab2D06	TGTAAGATGCCCACATT -F TCTCAGGCTTCAGGG – R	5	2.92	0.47	0.66

Number of alleles per locus (A), observed heterozygosity (Ho), effective alleles (Ae) and polymorphic information content (PIC).
Source: Author's computation.

Kogi and Lasiri also from Oyo either shared genetic pedigree with it or are its mutants. Similarly, released variety TDr95/19158 is closely related with farmer's accession Ajidawa from Oyo. In cluster 5, TDr89/02461

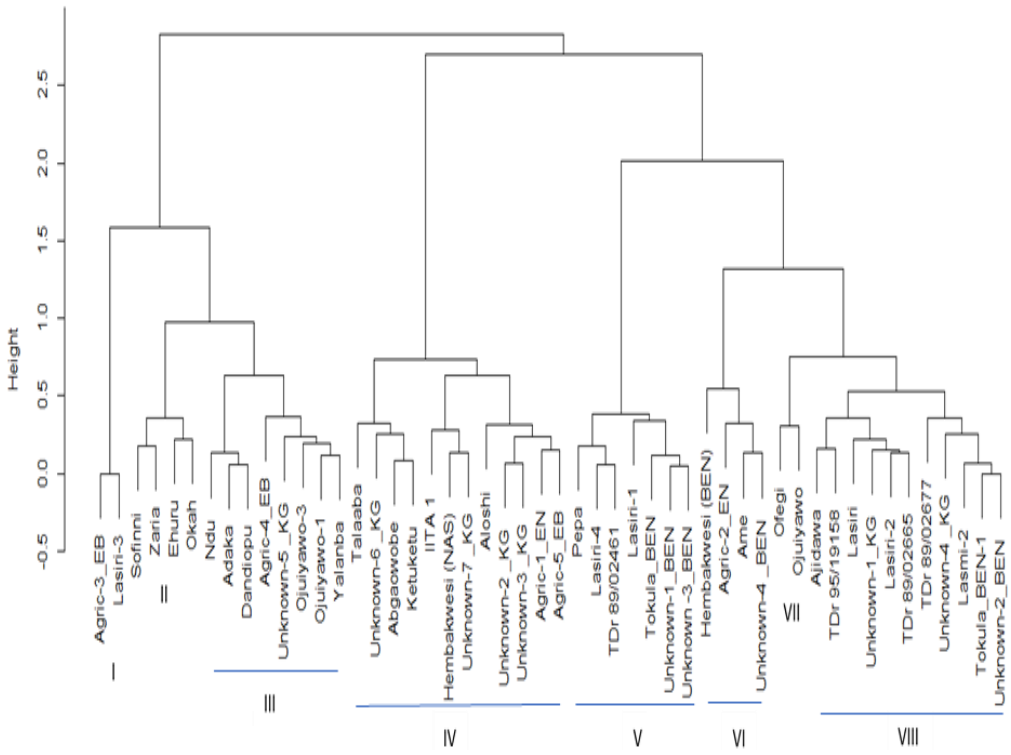

Figure 2. Dendrogram for the 52 *D. rotundata* cultivars constructed from SSRs data analysis using Unweighted Pair-group Arithmetic Average similarity matrices computed according to Nei coefficients.
Source: Author's computation.

is closely related to Lasiri-4 from Oyo and pepa form Nasarawa, indicating that both Lasiri-4 from Oyo and Pepa from Nasarawa might be the same as released variety TDr89/02461, but with different farmers' names at different locations. Also, there are strong relationships among the farmers' varieties from different locations, indicating that some of these might be the same but with different names at different locations.

In Figure 3, the 17 *D. alata* were classified into four main groups. Two of the three released varieties were in cluster 3; again, indicating that both of them might have the same genetic pedigree, while the third one was in cluster 4 indicating that it is more genetically distant from the previous two. In cluster 3, the two released varieties, TDa98/01176 and TDa00/00194 were in the same level of linkage with Tokula-2 and Local-3 all from Benue, implying that these accessions could be the same as either TDa98/0076 or TDa00/00194. Cluster 4 indicates that released variety TDa98/01168 is closely related to Agric-2 and local-1 from Oyo and Kogi, respectively. The accessions could also be thesame but named differently by farmers at different locations, similar to Agric-1 and Tokula-1 also from Oyo and Benue, respectively, which are of similar accessions but with different names at different locations; all mutants of TDa98/01168. The name "Agric" given to the accession in Oyo further buttresses the fact that it is a released variety as most improved crop accessions is commonly given such name by Nigerian farmers especially when it out-performs their local accessions.

Conclusion

The study revealed significant adoption of the released varieties in Nigeria, which have been renamed by farmers at different locations. Hence, there is a need for pragmatic paradigm shift in breeding approach, to make it more farmer-participatory. These will allow farmers, especially those who participated in the final evaluation (verification trials), to be involved in the final naming of the yet-to-be released varieties. Although this approach is already being deployed by the breeding programmes in the country with the aim that it will ease released variety identification and improve adoption efficiency. Also, the study equally revealed that adoption of these released varieties took place at Oyo, Kogi, Nasarawa and Benue axis and not in any other part of the country. This is expected as Oyo is the epicenter of yam breeding activities in Africa with the siting of IITA, while Kogi, Nasarawa and Benue are the yam production hotspots in the country where advanced breeding lines are evaluated.

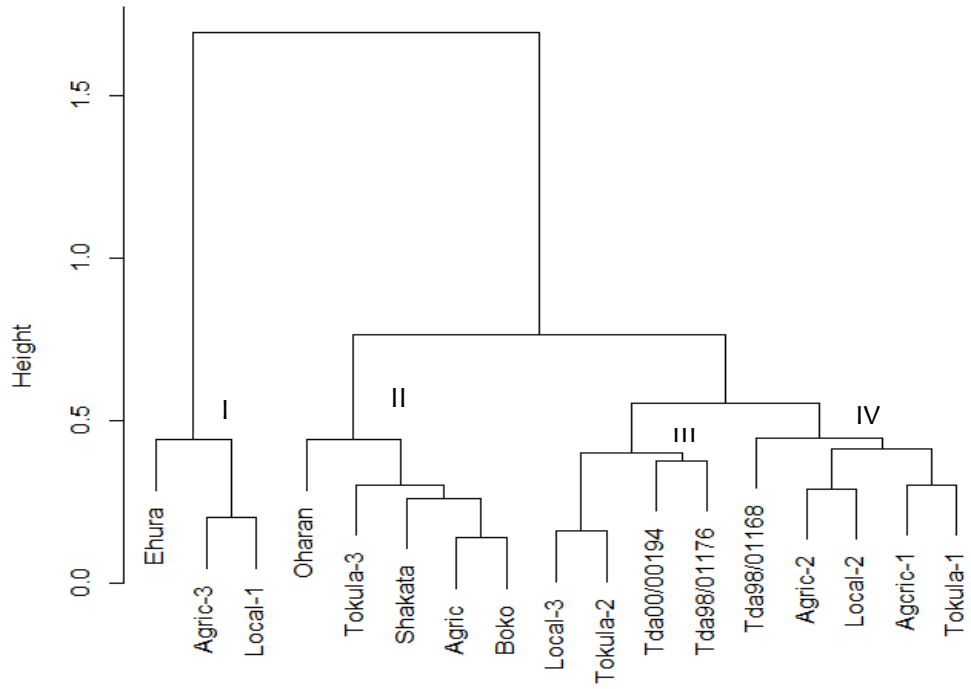

Figure 3. Dendrogram of 17 *D.alata* accessions developed from micro-satellite data using unweighted pair group of arithmetic means (UPGMA) based on Nei's (1978) genetic distance.

The implication here is that outside the farmers who participated in advanced evaluation of these released varieties, it is likely that other farmers, especially from outside the evaluation regions where yam production also takes place, have adopted the varieties so far released.

In addition, some of the farmers' accessions from different locations linked closely with each other; an indication that these accessions are similar, but differently named by farmers at different locations. Hence, this study's findings show the efficiency of SSRs used and the importance of using molecular markers in adoption studies.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

REFERENCES

Alene AD, Manyong VM (2007). The effects of Education on agricultural productivity under traditional and improved technology in northern Nigeria: an endogenous switching regression analysis. Empirical Economics 32(1):141-159.

Alene AD, Hassan RM (2006). The Efficiency of Traditional and Hybrid Maize Production in Eastern Ethiopia: An Extended Efficiency Decomposition Approach. Journal of African Economies 15(1):91-11

Ayensu ES, Coursey DG (1972) 'Guinea yams the botany, ethnobotany, use and possible future of yams in West Africa'. Economic Botany 26(4):301-318.

Food and Agricultural Organisation of the United Nations (FAO) (2018). Statistics, Division. Report. Available at: http://www.fao.org/faostat/en/#data/QC

Maass BL, Becker H, Oli MT (2007). Genetic Diversity in Yam Germplasm from Ethiopia and Their Relatedness to the Main Cultivated Species Assessed by AFLP Markers Genetic Diversity in Yam Germplasm from Ethiopia and Their Relatedness to the Main Cultivated Dioscorea Species Assessed by AFLP. Crop Science 47(4):1747-1753.

Nabeshima EH, Moro TMA, Campelo PH, Sant'Ana AS, Maria Teresa PS, Clerici MTPS (2020). Tubers and roots as a source of prebiotic fibers. Advances in Food and Nutrition Research 94:267-293. Available at: https://doi.org/10.1016/bs.afnr.2020.06.005

Nei M (1978). Estimation of average heterozygosity and genetic distance from a small number of individuals. Genetics 89(3):583-590.

Nweke FI, Robert A, Okoye B (2013). Yam consumption patterns in West Africa. Unpublished report prepared for Bill and Melinda Gates Foundation. Available at: https://doi.org/10.21955/gatesopenres.1115430.1

Obidiegwu JE, Asiedu R, Ene-Obong EE, Muonek CO, Kolesnikova-Allen M (2009b). Genetic characterization of some water yam (Dioscorea alata L) accessions in West Africa with simple sequence repeats Genetic characterization of some water yam (Dioscorea alata L) accessions in West. Journal of Food, Agriculture & Environment 7(3&4):634-638. 2009.

Obidiegwu JE, Akpabio EM (2017). The geography of yam cultivation in southern Nigeria; Exploring its meanings and cultural functions. Journal of Ethnic Foods 4(1):28-35.

Obidiegwu JE, Muoneke CO, Asiedu R. (2009c). SSR markers reveal diversity in Guinea yam (Dioscorea cayenensis / D. rotundata) core set. African Journal of Biotechnology 8(12):2730-2739.

Scarcelli N, Cubry P, Akakpo R, Anne-Celine T, Obidiegwu J, Baco MN, Otoo E, Sonke B, Dansi A, Djadatin G, Mariac C, Couderc M, Causse S, Alix K, Chair H, Francois O, Vigouroux Y (2019). Yam genomics supports West Africa as a major cradle of crop domestication. Science Advances 5(5):eaaw1947.

Sharma K, Mishra AK, Misra RS (2008). A simple and efficient method for extraction of genomic DNA from tropical tuber crops. African

Journal of Biotechnology 7(8):1018-1022.
Silva DM, Siqueira MVBM, Carrasco NF, Montello CC, Nascimnto WF, Veasey EA (2016). Genetic diversity among air yam (Dioscorea bulbifera) varieties based on single sequence repeat markers. Genetics and Molecular Research 15(2):15027929.
Siqueira MVBM, Marconi TG, Bonatelli ML, Zucchi MI, Veasey EA (2011). New microsatellite loci for water yam (Dioscorea alata, Dioscoreaceae) and cross-amplification for other Dioscorea species', American Journal of Botany 98(6):144-146.
Yu S, Darkwa K, Yaegashi H, Natsume S, Shimizu M, Abe A, Hirabuchi A, Ito K, Oikawa K, Tamiru-Oli M, Ohta A, Matsumoto R, Paterne A, De Koeyer D, Babil P, Yamanaka S, Muranaka S, Takagi H, White B, Asiedu R, Innan H, Asfaw A, Adebola P, Terauchi R (2020). Genome analyses reveal the hybrid origin of the staple crop white Guinea yam (Dioscorea rotundata). Proceedings of the National Academy of Sciences 117(50):31987-31992.
Tamiru M, Yamanaka S, Mitsuoka C, Babil P, Takagi H, Lopez-Montes A, Sartie A, Asiedu R, Terauchi R (2015). Development of genomic simple sequence repeat markers for yam', Crop Science 55(5):2191-2200.
Tostain S, Agbangla C, Scarcelli N, Mariac C, Dainou O, Berthaudi J, Pham J (2007). Genetic diversity analysis of yam cultivars (Dioscorea rotundata Poir.) in Benin using simple sequence repeat (SSR) markers. Plant Genetic Resources 5(2):71-81.

Vol. 21(5), pp.208-217, May 2022
DOI: 10.5897/AJB2021.17440
Article Number: E336DB069135
ISSN: 1684-5315

http://www.academicjournals.org/AJB

ACADEMIC JOURNALS
expand your knowledge

African Journal of Biotechnology

Full Length Research Paper

Efficient callus-mediated system for commercial production of sugarcane (*Saccharum* spp.) planting material in Ghana

Elaine Azu[1], Wilfred Elegba[1]*, Abigail Tweneboah Asare[1], Kwame Asare[1], Christian Akama[1], Patience Asare[1], Clement Annor[1], Samuel Azure[1] and Kenneth Danso[2]

[1]Biotechnology and Nuclear Agriculture Research Institute, Ghana Atomic Energy Commission, P. O. Box LG 80, Legon, Ghana.
[2]Graduate School of Nuclear and Allied Sciences, University of Ghana, P. O. Box AE1, Atomic, Accra, Ghana.

Received 7 December, 2021; Accepted 21 April, 2022

An efficient callus-mediated regeneration system was developed for high-frequency production of planting material of sugarcane genotypes LSC and B36464. Spindle leaf segments cultured on MS basal medium supplemented with 2,4-D or picloram at 1, 2, 3 or 4 mg/L resulted in callus induction. Callus induction was higher on 2,4-D amended medium compared to picloram. Nevertheless, for both auxins, callus induction improved significantly ($p \leq 0.05$) with increasing concentration; the highest (82 and 82.5% for B36464 and LSC respectively) was achieved at 4 mg/L. For shoot induction, calli were transferred to MS medium supplemented with BAP (0.1, 0.5, 1.0 or 1.5 mg/L). The highest number of shoots (18.13 and 16.75 for B36464 and LSC respectively) was achieved at 1.5 mg/L. Serial subculture at four-week intervals on a higher concentration of BAP (2.5 mg/L), in combination with NAA (0.5 mg/L) and GA_3 (0.5 mg/L), resulted in a four-fold increase in shoot number within 16 weeks. On this medium, 40% of shoot clusters of B36464 formed well-defined shoots. On MS medium containing solely NAA (3 mg/L), 88 and 72% (B36464 and LSC respectively) formed roots. Post-flask acclimatisation of the plantlets led to 85 and 91% survival rates in LSC and B36464 respectively after which plantlets were successfully transferred to field conditions. The callus-mediated regeneration system reported in this study has the potential to sustainably provide sugarcane planting material for the emerging sugar industry in Ghana.

Key words: Sugarcane, spindle leaf explants, callus-mediated organogenesis, plantlet regeneration, 2,4-D, Picloram.

INTRODUCTION

Globally, sugarcane (*Saccharum officinarum* L.) is an industrial crop used mainly to produce sugar and bioethanol. In Ghana, its full potential is yet to be exploited as most of the country's sugarcane is used either for the production of local alcoholic drink "*akpeteshie"* by small-scale distilleries or sold fresh as a snack (Ababio and Lovatt, 2015). Recently however, the crop is gaining more prominence in the country as an industrial crop particularly with the re-commissioning of the Komenda Sugar Factory in the Central Region of Ghana.

The sugar factory is expected to trigger large demand for planting materials for the establishment of plantations which in turn will serve as feedstock. Such plantations

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

may be established conventionally through vegetative propagation (planting of setts). However, setts are usually unavailable in commercial quantities and require high labour input for preparation and planting (Bello-Bello et al., 2018). Even when available, setts are often contaminated by pests and diseases resulting in low yields (Jalaja et al., 2008).

An alternative method of generating large quantities of planting material is tissue culture. Since the 1960s, the technique has been shown to be highly efficient for rapid multiplication of sugarcane (Heinz and Mee, 1969; Ho and Vasil, 1983). Plantlets have been successfully regenerated from various types of explants including immature leaf rolls (Soares et al., 2014), apical meristems (Ramgareeb et al., 2010), young leaves (Chengalrayan and Gallo-Meagher, 2001) and axillary buds (Vazquez Molina et al., 2005). Additionally, tissue culture has been shown to be advantageous in that, plants are disease-free thereby exhibiting increased field productivity and improved sugar yields (Sawant, 2014; Bello-Bello et al., 2018).

Although worldwide, significant progress has been made in refining tissue culture protocols for sugarcane, the crop has been shown to be genotype-dependent/ specific in its response to culture conditions (Jadhav et al., 2001). For instance, sugarcane genotypes can vary highly in their response to exogenous growth regulators in culture media (Gandonou et al., 2005). Consequently, efficient regeneration of specific genotypes often requires optimization of *in vitro* protocols (Tesfa and Ftwi, 2018).

Thus, in this study, we tested the response of two important local sugarcane cultivars to a simple and efficient callus-mediated *in vitro* regeneration system. This method of organogenesis not only presents a good strategy for rapid multiplication of the crop but also allows for future improvement of these cultivars via genetic transformation and *in vitro* mutagenesis (Jamil et al., 2017). For example, callus-mediated regeneration has been used to improve sugarcane for resistance to pests and diseases as well as tolerance to drought and salinity (Matsuoka et al., 2001; Eldessoky et al., 2011). Given the expected demand for large quantities of sugarcane planting material in Ghana, we envisage that the application of this technique to locally adapted and economically important cultivars has great potential to support the emerging sugar industry in the country. Additionally, it will contribute to job creation throughout-grower schemes, and improve productivity and income of smallholder farmers in sugarcane-growing regions.

MATERIALS AND METHODS

Study location

The experiment was conducted at the Biotechnology Centre of Biotechnology and Nuclear Agriculture Research Institute (BNARI), Ghana Atomic Energy Commission (GAEC) from March 2019 to September 2020.

Planting materials

Two genotypes of sugarcane, LSC and B36464 were used in the study. B36464 also locally referred to as *Alata*, is one of the main cultivars grown by smallholder farmers in the Komenda-Edina-Eguaafo-Abrem (KEEA) District of the Central Region, Ghana. It is also grown on commercial scale by farmers at Abura-Asebu-Kwamankese (AAK), Shama and Mpohor Wassa East districts in the Central Region due to their proximity to the Komenda Sugar Factory (GSS 2014; Yawson et al., 2018). The second genotype LSC is a popular landrace grown by smallholder farmers in several parts of Ghana including the Eastern, Central, Volta and Greater Accra regions. This landrace is often sold as a snack in small, peeled segments and provides income for many women in both rural and urban areas.

Explant preparation and sterilisation

Apical sections of field-grown canes (6 to 8 months old) were soaked overnight in 1% (w/v) fungicide solution (Bendazim® 50WP, active ingredient, carbendazim). Spindle leaf explants were obtained by removal of outer leaf whorls and trimming of the apical sections. The explants were then washed in mild detergent (Morning Fresh Dishwashing Liquid, Cussons®) solution for five minutes followed by soaking in 10% bleach (Clorox®, active ingredient – 6.05% sodium hypochlorite) for 30 min. Final surface sterilisation consisted of soaking explants in 0.2% (w/v) mercuric chloride for 10 min followed by thorough rinsing with three changes of sterile distilled water.

Callus induction

Thin slices (approximately 5 mm) of spindle leaf explants were inoculated on callus induction medium (CIM) which comprised Murashige and Skoog medium (1962) supplemented either with picloram or 2,4– dichlorophenoxyacetic acid (2,4 -D) at varying concentrations (0, 1, 2, 3 or 4 mg/L). The callus induction medium (CIM) also contained sucrose at 30 g/L. The pH of the culture medium was adjusted to 5.8 followed by addition of phytagel at 3.5 g/L and autoclaving at 121°C for 15 min. The cultures were maintained in the dark at 25 ± 1°C for 28 days, after which the number of explants that formed calli was scored. Fifty explants were cultured per treatment, consisting of 10 replicates.

Shoot induction from callus

For shoot induction, embryogenic calli (cream-coloured and friable) were transferred to shoot induction medium (SIM) consisting of MS medium supplemented with 6-benzylamino purine (BAP) at 0.1, 0.5, 1.0 or 1.5 mg/L, referred to hereafter as SIM1, SIM2, SIM3 and SIM4, respectively. The cultures were maintained under cool fluorescent light (16/8-h light/darkness; 2500 lux) at 25±1°C for six weeks after which the number of germinated shoots per callus clump was scored for the various treatments. Sixteen calli were cultured per treatment with each callus considered as a replicate.

Shoot proliferation and root induction

To improve shoot multiplication, micro shoot clusters were transferred to shoot proliferation media (SPM) containing BAP at 2.5 mg/L in combination with NAA (0.5 mg/L) and GA_3 (0.5 mg/L). The total number of well-differentiated shoots was scored at the end of each culture cycle (every four weeks). Multiplication rates were calculated as a ratio of the total number of shoots at each

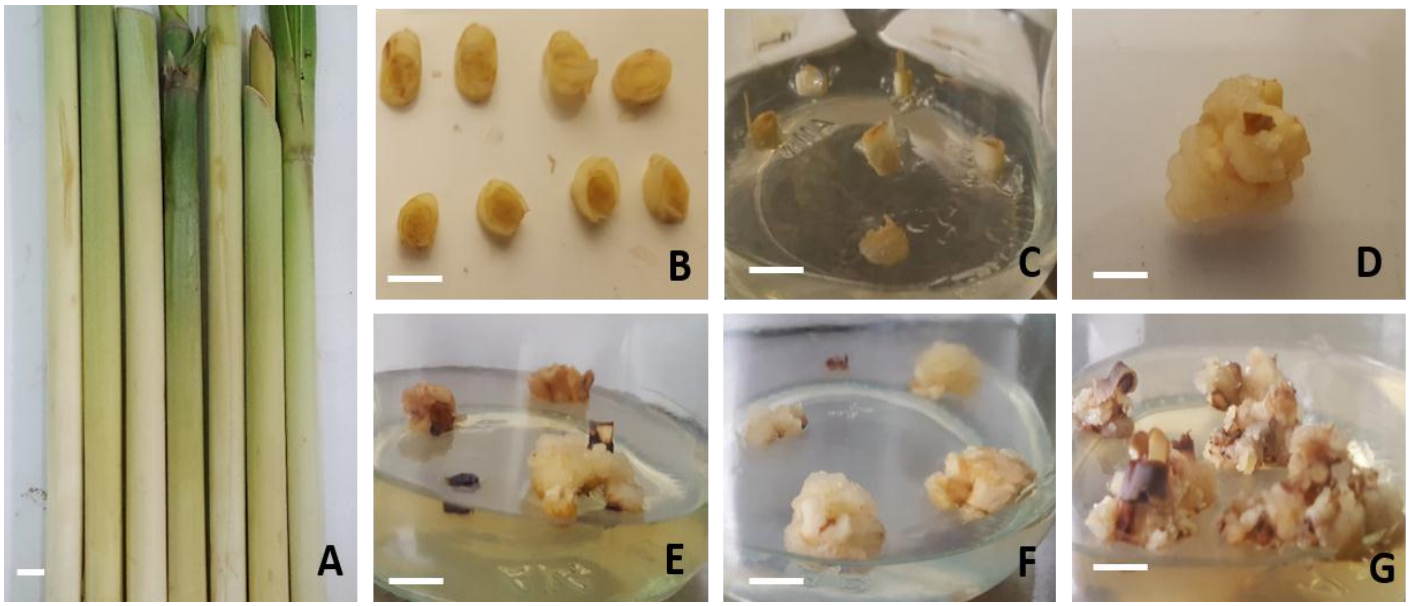

Figure 1. Callus induction from spindle leaf explants of LSC and B36464. **(A)** Spindle leaf explants **(B, C)** Sliced explants before and after inoculation **(D, E)** Callus formation in LSC explants incubated on MS media supplemented with 2,4-D and picloram respectively (**F, G)** Callus formation in B36464 incubated on MS media supplemented with 2,4-D and picloram respectively. Bar represents 1cm (**A**) and 5mm (**B - G)**.

sub-culture divided by the number of shoots at the previous subculture stage. Unrooted shoots were transferred to MS medium containing NAA at 3 mg/L (RIM) for root induction.

Plantlet acclimatization

Two hundred plantlets (100 per genotype) were washed gently with tap water followed by a five-minute rinse in 0.1% fungicide solution (Bendazim® 50WP, active ingredient, carbendazim). The plantlets were then transferred to polythene bags filled with soil, cocopeat and sawdust mixture (2:1:1) with high humidity conditions (>85%) created by covering the plantlets with transparent plastic cups for three days. The plantlets were maintained in a greenhouse at 28 ± 2°C and were irrigated every other day for six weeks. The number of plants that survived was recorded. Following successful acclimatisation, well-hardened plantlets (approximately 9 weeks old) were transferred to field conditions. Prior to transplanting, older leaves were trimmed. During transplanting, plantlets were carefully removed from polythene bags to minimise damage to roots. Plantlets were then placed in furrows two feet apart and covered with adequate amounts of soil and irrigated immediately after transplanting. All recommended intercultural operations (weeding, irrigation and pest control) were performed.

Statistical analysis

All experiments were laid out in a completely randomized design (CRD). For callus induction, each treatment was replicated 10 times. For shoot induction, each treatment was replicated 16 times. Data were analysed using Microsoft Excel 2010 and GraphPad Prism version 7 (San Diego, United States). All data were subjected to analysis of variance (ANOVA) and statistically significant results at the 5% level were compared either with Tukey's or Sidak's multiple comparisons test.

RESULTS AND DISCUSSION

Callus induction

Spindle leaf explants (Figure 1A) of sugarcane cultivars LSC and B36464 formed callus on MS media containing varying concentrations of 2,4-D or picloram by the twelfth day of incubation (Figure 1B, C). For both cultivars, the absence of 2,4-D and picloram in the culture media resulted in no callus production, indicating an auxin requirement for callus formation in sugarcane, similar to other plant species (Ikeuchi et al., 2013; Osman et al., 2016; Zang et al., 2016). Similar observations were made by Ramgareeb et al. (2010) and Alcantara et al. (2014) where various types of sugarcane explants failed to form callus on auxin-free media. Exogenous auxin supplementation is required to reprogram somatic cells to acquire pluripotency as well as initiate cell division leading to the formation of callus (Skoog and Miller, 1957; George et al., 2008; Fehér, 2019). An increase in the number and size of calli was observed after 21 days of incubation on both 2,4-D (Figure 1D, E) and picloram-supplemented media (Figure 1F, G).

Callus induction on 2,4-D supplemented media was significantly higher ($p \leq 0.05$) compared to picloram at the same concentrations (Table 1; supplementary Table 1) suggesting that 2,4-D is an effective auxin for callus

Table 1. Callus induction in LSC and B36464 sugarcane genotypes on 2,4-D or picloram-supplemented MS media after 21 days in the dark.

Auxin	Concentration (mg/l)	Mean callus production (%) ± SD	
		LSC	B36464
2,4-D	C (control)	0.0 ± 0.00[a]	0.0 ± 0.00[a]
	D1	18 ± 0.39[a]	20.0 ± 0.41[a]
	D2	44 ± 0.50[b]	72.5 ± 0.45[b]
	D3	68 ± 0.47[c]	77.5 ± 0.38[b]
	D4	82 ± 0.39[c]	82.5 ± 0.38[b]
Picloram	P1	10 ± 0.30[a]	7.5 ± 0.27[a]
	P2	16 ± 0.37[a]	22.5 ± 0.42[ac]
	P3	44 ± 0.50[bc]	27.5 ± 045[ac]
	P4	32 ± 0.47[bc]	60.0 ± 0.49[bc]

induction in sugarcane. This is similar to earlier reports(Ho and Vasil, 1983; Gill et al., 2004; Dibax et al., 2011; Alcantara et al., 2014; Arjun Srinath, 2015). However, our findings contradict those of Gallo-Meagher et al. (2012) who reported higher callus induction on picloram-supplemented medium rather than 2,4-D in sugarcane. This may be due to differences in genotypic response to auxins as reported by Gandonou et al. (2005) and Mekonnen et al. (2014).

In general, induction of embryogenic calli improved with increasing auxin concentration (Table 1). For LSC, callus induction was best achieved at the highest concentrations of 3 and 4 mg/L for 2,4-D. Similar observations have been reported by several authors including Badawy et al. (2008), Jahangir et al. (2010) and Arjun Srinath (2015). A similar trend was observed for picloram. However, as picloram concentration increased from 3 to 4 mg/L callus induction rate declined (from 44 to 34%) in LSC. The reduction in callus formation may be attributed to the presence of endogenous hormones in the explant tissues prior to culture initiation (Can et al., 2008). Wernicke et al. (1986), explain that high auxin concentrations may prevent meristematic cell divisions, resulting in decreased reactions such as callus induction. In the genotype B36464, the best concentration for callus formation is between 2- 4 mg/L 2,4-D or 4 mg/L picloram. Nonetheless, the response of the two genotypes in this study to callus induction is beneficial in that, it is a first and crucial step in *in vitro* mutagenesis as well as genetic transformation protocols for improvement of local sugarcane genotypes. Calli are ideal propagules for reception of genes since they are mostly of single cell origin (Nagmani et al., 1987) and can also be reached by either physical or chemical mutagens in mutagenesis (ref).

Mean production of embryogenic callus is the ratio of callus formed to the number of explants cultured expressed as a percentage. Data represent mean ± SD. Values in a column followed by the same letters are not significantly different from each other at $p \leq 0.05$ (Tukey's pairwise comparison test).

Shoot induction from calli

The addition of cytokinins, particularly BAP, to MS medium has been shown to be effective for inducing shoots from sugarcane callus cultures (Ather et al.. 2009; Dibax et al.. 2011; Arjun Srinath. 2015). Thus, to determine the optimum concentration of BAP for shoot induction from callus for the two cultivars, we transferred friable calli to MS medium containing BAP ranging from 0.1 to 1.5 mg/L BAP. By the second week, green microstructures were observed on the surfaces of calli of both B36464 (Figure 2A, B) and LSC (Figure 2C, D), suggesting the formation of totipotent shoot primordia. The presence of high concentration of cytokinin in the shoot induction medium induces shoot stem cell regulators from callus (Gordon et al., 2007). According to Dibax et al. (2013), the formation of such shoot primordia is triggered from both nodular and friable calli due to the presence of cytokinins which initiate a pathway redirection. The formation of shoot primordia on callus of sugarcane resulted in the development of shoots within 28 days of culture. The mean number of shoots formed per callus explants ranged from 9.69 ± 2.55 to 18.13 ± 3.07 in B36464 genotype and 8.44 ± 4.19 to 16.75 ± 3.55 in LSC genotype (Figure 3; Supplementary Table 2). The shoot induction rates obtained from callus cultures in this study are higher than those obtained from shoot tip or lateral bud culture (Danso et al., 2011; Mekonnen et al., 2014) making this protocol efficient for commercial production of sugarcane planting materials. Callus develops more shoot primordium, hence more shoots than from direct organogenesis in shoot tips. Shoot regeneration from callus is a result of localised cell division leading to differentiation of globular meristemoids which can develop into shoots (Ovečka et al., 1997). For

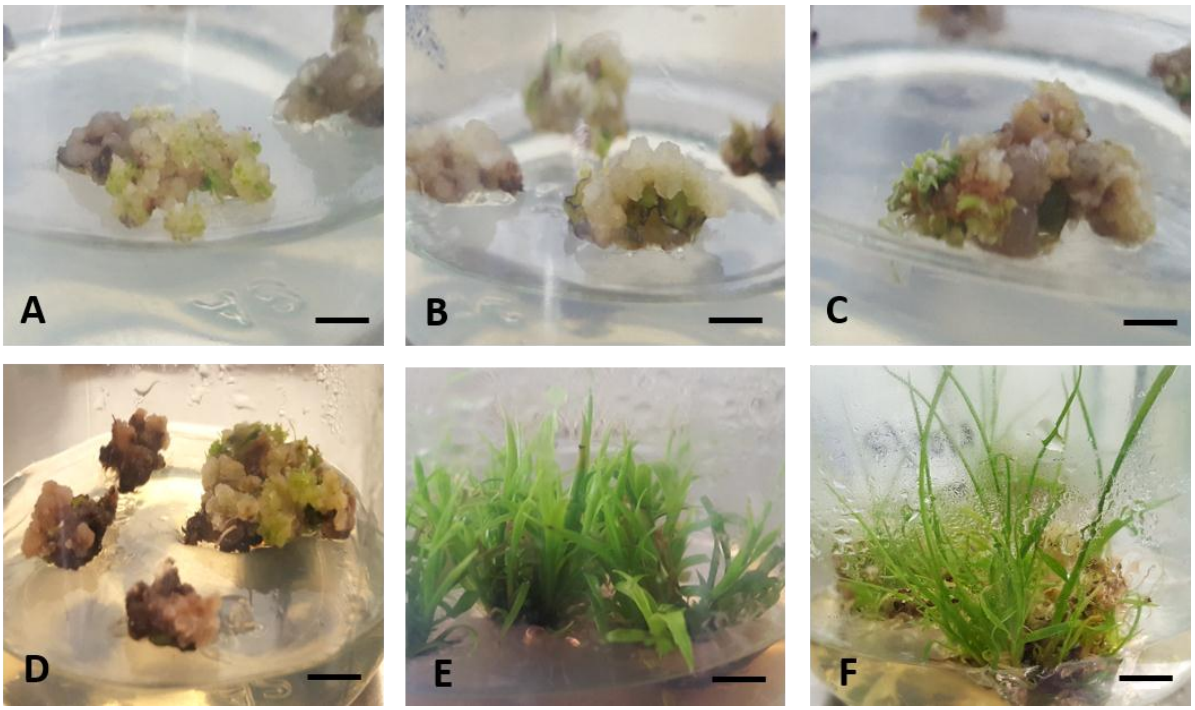

Figure 2. Shoot induction from calli of sugarcane genotypes B36464 and LSC. **(A, B)** Green micro shoots forming on embryogenic callus of B36464 and **(C, D)** LSC after three weeks of incubation. Shoot clusters of **(E)** B36464 and **(F)** LSC respectively after six weeks of incubation. Bar represents 2mm (**A - D)** and 1mm (**E, F)**.

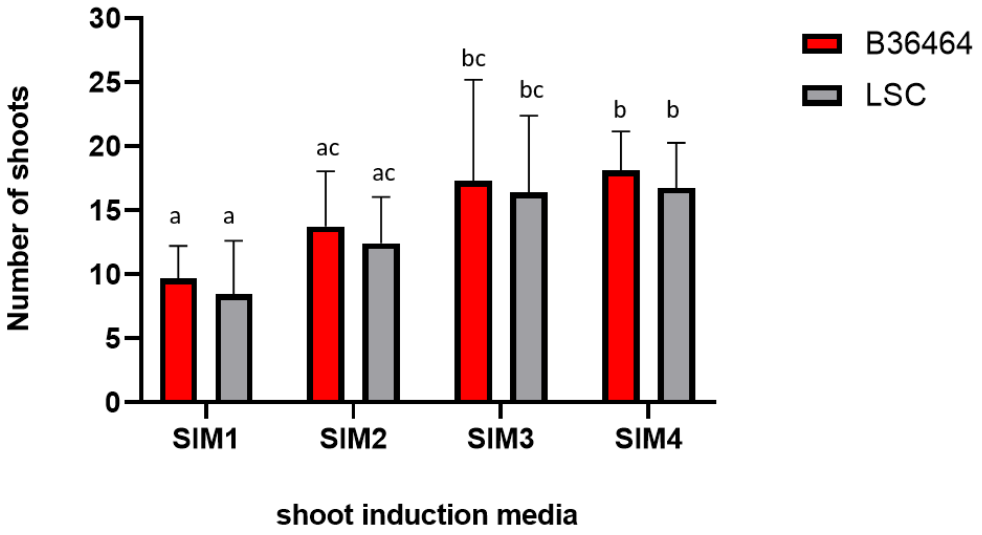

Figure 3. Shoot induction in calli of LSC and B36464 genotypes on MS media with varying concentrations of BAP after 4 weeks of culture.

example, in *Nicotiana tabacum,* the palisade cells divide to form callus which gives rise to shoot primordia and finally regeneration into shoot (Gupta et al., 1966; Zhi-hong and Gui-Yun, 1980).

The lowest concentrations of BAP (0.1 and 0.5 mg/L) induced the least number of shoots from callus in both genotypes. However, at higher BAP concentrations of 1.0 and 1.5 mg/L, we recorded a significant increase ($p \leq 0.05$) in shoot induction in both genotypes (Supplementary Table 2). An increase in shoot production

Table 2. Shoot multiplication rates in sugarcane cultivars LSC and B36464 on SPM at 4-week intervals.

Sub-culture cycle (weeks)	Total number of shoots		Multiplication rates	
	LSC	B36464	LSC	B36464
4	2902	2797		
8	6927	7209	2.39	2.58
12	9673	10724	1.40	1.49
16	12278	13707	1.27	1.28
Total	12278	13707	4.23	4.90

from sugarcane callus cultures with increasing BAP concentration has been reported by Behera and Sahoo (2009) and Hapsoro (2017). A comparison between the two genotypes showed higher shoot induction from callus of variety B36464 compared with LSC, suggesting genotype effect on shoot induction. Similarly, genotypic differences have been observed in shoot regeneration from callus cultures of different sugarcane cultivars (Gandonou et al., 2005; Kaur and Kapoor, 2016; Di Pauli et al., 2021).

Shoot proliferation and root induction

Given the increasing frequency of shoot induction (from callus) with increasing BAP concentration in the previous experiment, we evaluated the proliferation of shoots on MS medium supplemented with a higher concentration of BAP (2.5 mg/L) in combination with low concentrations of NAA (0.1 mg/L) and GA_3 (0.1 mg/L). In sugarcane, as in many plant species, a high cytokinin to auxin ratio in the culture medium has been shown to effectively stimulate shoot regeneration and differentiation (Ikeuchi et al., 2013; Fehér, 2019). In order to increase plantlet production and maximise economic gains, four serial subcultures were used to multiply the shoots in culture.

After subculture cycle 1, the total number of shoots produced in LSC genotype was higher (2902) compared to B36464 (2797) (Table 2 and Figure 4A, B). However, after 8 weeks of culture on SPM (cycle 2), shoot production was higher (7209) in B36464 compared to LSC (6927) and this trend was maintained for the next two cycles (till 16 weeks of culture). The total number of shoots formed in both genotypes increased more than two-fold by the second subculture (at 8 weeks) and four-fold by the fourth subculture (at 16 weeks) (Table 2). This high frequency of plantlet production indicates the efficiency of the callus-mediated technique for sugarcane micro-propagation.

Interestingly, shoot multiplication rates decreased with successive subcultures (second subculture onwards) likely due to the cumulative effects of BAP in succeeding subcultures which might have led to high endogenous concentration of BAP, hence the phytotoxic effect. In general, decline in shoot multiplication rates as a result of repeated *in vitro* subculture has been reported in several plant species (Norton and Norton, 1986; Hussain et al., 2007; Vujović et al., 2012). Although reducing the concentration of hormones in the culture medium can delay the decline in shoot multiplication rates (Vujović et al., 2012), regular initiation of new cultures from spindle leaf explants is necessary to maintain high shoot multiplication rates. The initiation of new cultures is important to reduce the risk of somaclonal variation which is quite common in sugarcane (Khan, 1999; Khan et al., 2008). It also ensures genetic homogeneity of plantlets and the production of true-to-type planting materials for the establishment of commercial plantations (Petolino et al., 2003). However, in this work using LSC and B36464 genotypes, we did not observe any genetic or somaclonal variation in plants transferred to the field at six months old.

With respect to root induction, 25% of shoot clusters of LSC formed well-defined roots spontaneously on BAP-containing medium (SPM) after 4 weeks of culture compared to 57% in B36464 shoot clusters (Figure 4C). This phenomenon of spontaneous root formation at the shoot maturation stage on media containing only cytokinins has been reported by Zamir et al. (2012). Nonetheless, to improve root formation from *in vitro* shoots, we supplemented MS media with 3 mg/L NAA which resulted in marked improvement in root development in both B36464 (88%) and LSC (72%) after 8 weeks of culture.

Plantlet acclimatization

Efficient weaning and hardening of *in vitro*-generated plantlets are critical for successful transfer of plantlets to field conditions. To determine the efficiency of weaning and hardening, a total of 200 plantlets (100 per genotype) were transferred to the greenhouse for weaning. Survival rates of 85 and 91% were achieved for LSC and B36464, respectively (Figure 5). The rates of acclimatization obtained for plantlets of both sugarcane genotypes in this study are similar to survival rates obtained for other commercial Indian varieties (Kaur and Kapoor, 2016) but higher compared to other reports where up to 87% survival rates were obtained (Mekonnen et al., 2014). Well-developed roots in *in vitro* plantlets prior to weaning

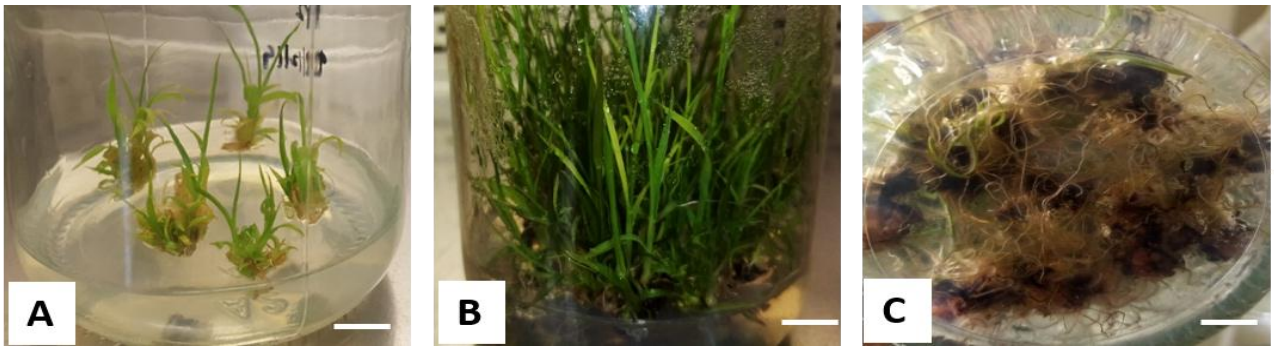

Figure 4. Shoot proliferation and root induction in B36464.
(A) Separated micro shoot clusters on SPM **(B)** Shoot proliferation and **(C)** root induction after 4 weeks of culture. Bar represents 1 mm.

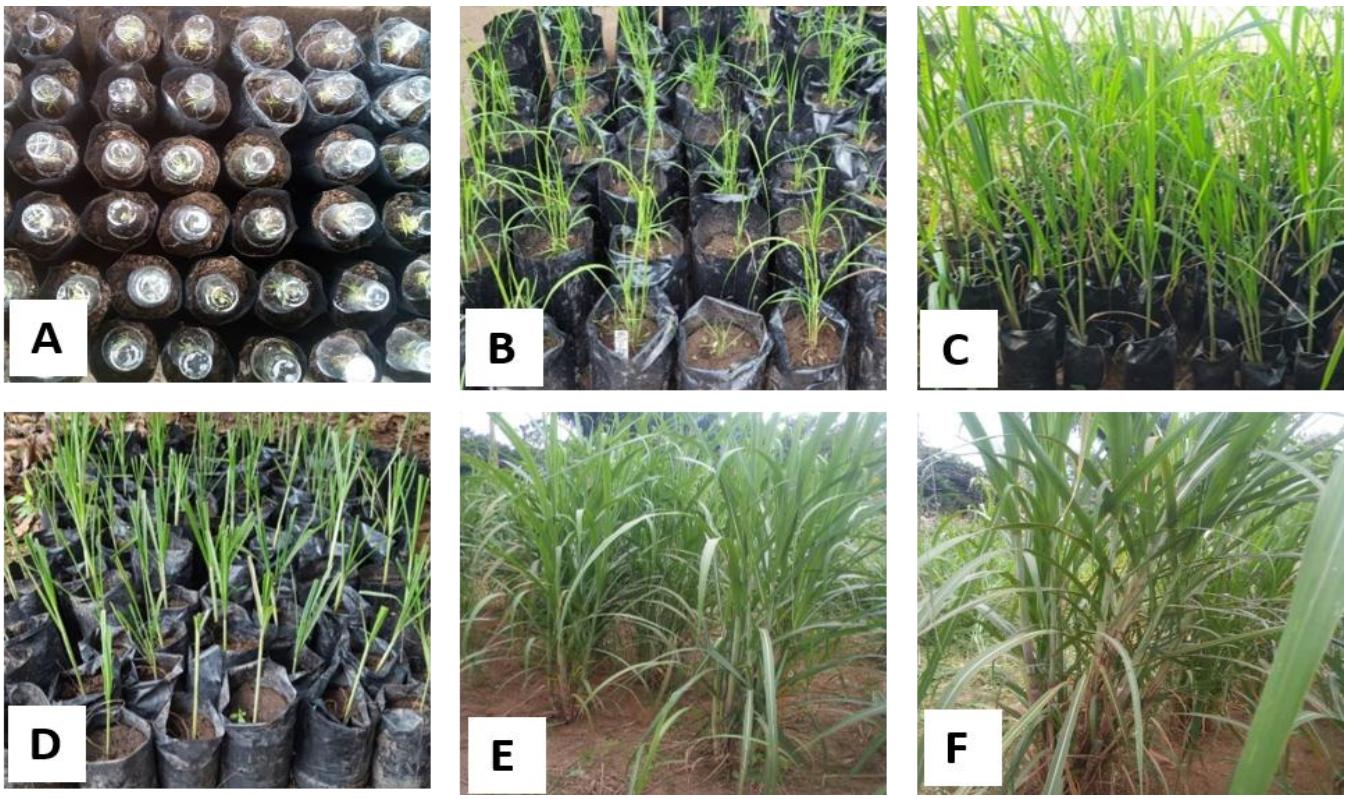

Figure 5. Plantlet acclimatization and field establishment of B36464 variety.
(A) Weaned plantlets in humidity chamber. Weaned plantlets at **(B)** 2-weeks and **(C)** 8 weeks old. **(D)** Older leaves of weaned plantlets trimmed prior to field transfer. Established plants at **(E)** three months and **(F)** six months after transfer to the field.

have been shown to enhance survival of plants at the weaning stage (Danso et al., 2011). Other factors such as genotype and the medium used for weaning also play significant role in post-flask acclimatisation: cocopeat: soil: sawdust mixture (2:1:1) could explain the differences in plantlet acclimatization compared with other studies. The weaned plantlets were successfully transferred to field after 8 weeks of hardening for further development.

Conclusion

The emerging sugar industry in Ghana and across Africa requires the availability of large quantities of sugarcane planting materials to ensure sustainability of the industry. As a strategy for meeting this demand, the response of two important locally adapted cultivars to micro-propagation via somatic embryogenesis was tested. The

successful induction of embryogenic calli from spindle leaf explants in the two cultivars followed by efficient plantlet regeneration on cytokinin-auxin amended MS medium augur well for adequate and sustainable supply of planting materials for the sugarcane industry. A four-fold increase in shoot production in 16 weeks demonstrates the efficiency of our protocol for large scale production of sugarcane planting material. Thus, this study has demonstrated that somatic embryogenesis using spindle leaf explants offers an efficient protocol for large scale production of disease-free sugarcane plantlets for the emerging sugar industries across Africa.

ABBREVIATIONS

2,4-D, 2,4-dichlorophenoxyacetic acid; **BAP,** 6 – benzylamino purine; **BNARI,** Biotechnology and Nuclear Agriculture Research Institute; **GAEC,** Ghana Atomic Energy Commission; **MS,** Murashige and Skoog Basal medium; **NAA,** 1-naphthaleneacetic acid; **Picloram,** 4-amino-3,5,6-trichloropicolinic acid.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENTS

The authors are grateful to the management of BNARI (GAEC) for the use of laboratory space and provision of resources necessary for this experiment and wish to express gratitude to the entire staff of the Biotechnology Centre for their diverse contributions to this work. The authors appreciate Mr. Nehemiah Naanie and Mr. Owula for their enormous assistance in the maintenance of the sugarcane germplasm on station. This study was funded by the government of Ghana through the Biotechnology and Nuclear Agriculture Research Institute (BNARI), Ghana Atomic Energy Commission.

REFERENCES

Ababio P, Lovatt P (2015). A review on food safety and food hygiene studies in Ghana. Food Control 47:92-97.

Alcantara GBd, Dibax R, Oliveira RAd, Bespalhok Filho JC, Daros E (2014). Plant regeneration and histological study of the somatic embryogenesis of sugarcane (*Saccharum* spp.) cultivars RB855156 and RB72454. Acta Scientiarum. Agronomy 36(1):63-72.

Arjun Srinath R (2015). Callus Induction and Organogenesis in Sugarcane (*Saccharum officinarum* L.) var 93v297. International Letters of Natural Sciences 48:14-22.

Ather A, Khan S, Rehman A, Nazir M (2009). Optimization of the protocols for callus induction, regeneration and acclimatization of sugarcane cv. Thatta-10. Pakistan Journal of Botany 41(2):815-820.

Badawy O, Nasr M, Alhendawi R (2008). Response of sugarcane (*Saccharum* species hybrid) genotypes to embryogenic callus induction and in vitro salt stress. Sugar Tech 10(3):243-247.

Behera K, Sahoo S (2009). Rapid *In vitro* Micro propagation of Sugarcane (*Saccharum officinarum* L. cv- Nayana) Through Callus Culture. Nature and Science 7(4):1-10.

Bello-Bello J, Mendoza-Mexicano M, Perez Sato JA (2018). In Vitro Propagation of Sugarcane for Certified Seed Production. In Sugarcane-Technology and Research. IntechOpen.

Can E, Çeliktaş N, Hatipoğlu R (2008). Effect of Auxin Type and Concentrations in Different Media on the Callus Induction and Shoot Formation of Crested Wheatgrass (*Agropyron Cristatum* (L.) Gaertn). Biotechnology & Biotechnological Equipment 22(3):782-786.

Chengalrayan K, Gallo-Meagher M (2001). Effect of various growth regulators on shoot regeneration of sugarcane. *In Vitro* Cellular and Developmental Biology-Plant 37(4):434-439.

Danso KE, Azu E, Elegba W, Asumeng A, Amoatey HM, Klu G (2011). Effective decontamination and subsequent plantlet regeneration of sugarcane (*Saccharum officinarum* L.) *in vitro*. International Journal of Integrative Biology 11(2):90-96.

Di Pauli V, Fontana PD, Lewi DM, Arturo F, Erazzu LE (2021). Optimized somatic embryogenesis and plant regeneration in elite Argentinian sugarcane (*Saccharum spp*.) cultivars. Journal of Genetic Engineering and Biotechnology 19(1):1-8.

Dibax R, Alcantara G, Machado M, Filho JCB, oliveira RA (2013). Protocol optimization and histological analysis of *in vitro* plant regeneration of 'RB92579' and 'RB93509' sugarcane cultivars. Ciência Rural 43:49-54.

Dibax R, Alcantara G, Bespalhok J, Machado M, Oliveira Y, Lopes da Silva AL (2011). Plant regeneration of sugarcane cv. RB931003 and RB98710 from somatic embryos and acclimatization. Journal of Biotechnology and Biodiversity 2(3):32-37.

Eldessoky DS, Ismail RM, Abdel-Hadi AH, Abdallah NA (2011). Establishment of regeneration and transformation system of sugarcane cultivar GT54-9 (C9). GM Crops 2(2):126-134.

Fehér A (2019). Callus, Dedifferentiation, Totipotency, Somatic Embryogenesis: What These Terms Mean in the Era of Molecular Plant Biology? Frontiers in plant science 10:536.

Ghana Statistial Service (GSS) (2014). 2010 Population and Housing Census. District Analytical Report Ghana Statistial Service.

Gallo-Meagher M, English R, Abouzid A (2012). Thidiazuron stimulates shoot regeneration of sugarcane embryogenic callus. In Vitro Cellular and Developmental Biology-Plant 36(1):37-40.

Gandonou C, Errabii T, Abrini J, Idaomar M, Chibi F, Skali-Senhaji N (2005). Effect of genotype on callus induction and plant regeneration from leaf explants of sugarcane (*Saccharum* sp.). African Journal of Biotechnology 4(11):1250-1255

George EF, Hall MA, De Glerk G (2008). Plant propagation by tissue culture vol 1. Somatic embryogenesis Springer, New York, USA.

Gill N, Gill R, Gosal S (2004). Factors enhancing somatic embryogenesis and plant regeneration in sugarcane (*Saccharum officinarum* L.). Indian Journal of Biotechnology 3:119-123.

Gordon SP, Heisler MG, Reddy GV, Ohno C, Das P, Meyerowitz EM (2007). Pattern formation during de novo assembly of the Arabidopsis shoot meristem. Development 134(19):3539-3548.

Gupta G, Guha S, SC M (1966). Differentiation of buds from leaves of *Nicotiana tabacum* Linn. in sterile culture. Phytomorphology 16:175-182.

Hapsoro D (2017). In Vitro Shoot Formation on Sugarcane (*Saccharum officinarum* L.) Callus as Affected by Benzyladenine Concentrations. Indonesian Journal of Agronomy 40(1):56-61.

Heinz D, Mee G (1969). Plant Differentiation from Callus Tissue of Saccharum Species1. Crop Science 9(3):346-348.

Ho WJ, Vasil IK (1983). Somatic embryogenesis in sugarcane (*Saccharum officinarum* L.) I. The morphology and physiology of callus formation and the ontogeny of somatic embryos. Protoplasma 118(3):169-180.

Hussain TM, Chandrasekhar T, Gopal GR (2007). High frequency shoot regeneration of *Sterculia urens* Roxb. an endangered tree species through cotyledonary node cultures. African Journal of Biotechnology 6(4):1643-1649.

Ikeuchi M, Sugimoto K, Iwase A (2013). Plant callus: mechanisms of induction and repression. Plant Cell 25(9):3159-3173.

Jadhav AB, Vaidya E, Aher VB, Pawar AM (2001). In-vitro multiplication of 'Co-86032' sugarcane (*Saccharum officinarum*) hybrid. The Indian

Journal of Agricultural Sciences 71(2):113-115.
Jahangir G, Nasir I, Sial R, Javid M, Husnain T (2010). Various Hormonal Supplementations Activate Sugarcane Regeneration In-vitro. Journal of Agricultural Science 2(4):231.
Jalaja N, Neelamathi D, Sreenivasan T (2008). Micropropagation for Quality Seed Production in Sugarcane in Asia and the Pacific. Volume 1. Food and Agriculture Organization of the United Nations.
Jamil S, Shahzad R, Talha GM, Sakhawat G, Sajid ur R, Sultana R, Iqbal MZ (2017). Optimization of Protocols for In Vitro Regeneration of Sugarcane (*Saccharum officinarum*). International Journal of Agronomy 2017:2089381.
Kaur R, Kapoor MP (2016). Plant Regeneration Through Somatic Embryogenesis in Sugarcane. Sugar Tech 18(1):93-99.
Khan HU (1999). Somaclonal variation in sugarcane through tissue culture and subsequent screening for salt tolerance. Department of Plant Breeding and Genetics, Faculty of Agriculture, Gomal University, Dera Ismil Khan.
Khan I, Dahot M, Seema N, Sajida B, Khatri A (2008). Genetic variability in plantlets derived from callus culture in sugarcane. Pakistan Journal of Botany 40(2):547-564.
Matsuoka M, Ideta O, Tanio M, Hayakawa A, Miwa H (2001). Agrobacterium tumefaciens-mediated transformation of sugarcane using cell suspension culture with a novel method. Paper presented at the International Society of Sugar Cane Technologists. Proceedings of the XXIV Congress, Brisbane, Australia pp. 17-21 September 2001. Australian Society of Sugar Cane Technologists. 2:660-662.
Mekonnen T, Diro M, Manju S, Tadesse N (2014). Protocol optimization for in vitro mass propagation of two sugarcane (*Saccharum officinarum* L.) clones grown in Ethiopia. African Journal of Biotechnology 13(12):1358-1368.
Nagmani R, Becwar M, Wann S (1987). Single-cell origin and development of somatic embryos in *Picea abies* (L.) Karst. (Norway spruce) and P. glauca (Moench) Voss (white spruce). Plant Cell Reports 6(2):157-159.
Norton ME, Norton CR (1986). Change in shoot proliferation with repeated in vitro subculture of shoots of woody species of Rosaceae. Plant Cell, Tissue and Organ Culture 5(3):187-197.
Osman NI, Jaafar Sidik N, Awal A (2016). Effects of variations in culture media and hormonal treatments upon callus induction potential in endosperm explant of *Barringtonia racemosa* L. Asian Pacific Journal of Tropical Biomedicine 6(2):143-147.
Ovečka M, Bobák M, Šamaj J (1997). Development of shoot primordia in tissue culture of *Papaver somniferum* L. Biologia Plantarum 39(4):499-506.
Petolino JF, Roberts JL, Jayakumar P (2003). Plant cell culture. In: Handbook of Industrial Cell Culture. Springer pp. 243-258.
Ramgareeb S, Snyman S, Van Antwerpen T, Rutherford R (2010). Elimination of virus and rapid propagation of disease-free sugarcane (Saccharum spp. cultivar NCo376) using apical meristem culture. Plant Cell, Tissue and Organ Culture 100(2):175-181.
Sawant R (2014). Sawant RA,Tawar PN, Meti NT, Ranjekar PK (2014). Role of sugarcane micropropagation for production of quality seed. International Journal of Recent Biotechnology 2(4):34-41.
Skoog F, Miller CO (1957). Chemical regulation of growth and organ formation in plant tissues cultured in vitro. Symposia of the Society for Experimental Biology 11:118-130.
Soares RR, Ferreira ME, Gamarano MC, Ribeiro RC, Sabino VM, Pereira BMH (2014). The use of histological analysis for the detection of somatic embryos in sugarcane. African Journal of Biotechnology 13(6):762-767.
Tesfa M, Ftwi M (2018). In Vitro Plant Regeneration of Sugarcane (*Saccharum spp*.) Variety Inoculated Under Different Levels of Plant Growth Regulators. Journal of Plant Biochemistry and Physiology 6(4):1000227.
Vazquez Molina DE, De Los Santos A, Lecona Guzman KA, Sumano Muniz O, Velazquez Mendez M, Rincon Rosales R, Llaven O, Dendooven L, Gutierrez-Miceli FA (2005). Sugar cane buds as an efficient explant for plantlet regeneration. Biologia Plantarum 49(4)481-485.
Vujović T, Ruzic D, Cerović R (2012). In vitro shoot multiplication as influenced by repeated subculturing of shoots of contemporary fruit rootstocks. Horticultural Science 39(3):101-107.
Wernicke W, Grost J, Molkovits L (1986). The ambiguous role of 2,4-dichlorophenoxyacetic acid in wheat tissue culture. Physiologia Plantarum 68(4):597-602.
Yawson DO, Adu MO, Osei KN (2018). Spatial assessment of sugarcane (Saccharurn spp. L.) production to feed the Komenda Sugar Factory, Ghana. Heliyon 4(11):e00903.
Zamir R, Khalil, Shah, Khan M, Khan NA, Shahenshah (2012). Efficient In Vitro Regeneration of Sugarcane (*Saccharum Officinarum* L.) from Bud Explants. Biotechnology and Biotechnological Equipment 26(4):3094-3099.
Zang Q, Zhou L, Zhuge F, Yang H, Wang X, Lin X (2016). Callus induction and regeneration via shoot tips of *Dendrocalamus hamiltonii*. SpringerPlus 5(1):1-7.
Zhi-hong X, Gui-Yun L (1980). Histo-Cytological Observations on Callus and Bud Formation in Cultures of *Nicotiana tabacum* L. Journal of Integrative Plant Biology 22(1).

Supplementary Table 1. Two-way ANOVA table comparing callus formation from spindle leaf explants on 2,4-D and picloram supplemented media after 21 days of incubation in the dark.

Source of variation	% of total variation	P value	P value summary	Significance	
Interaction	2.787	0.0642	ns	No	
Cultivars	67.21	<0.0001	****	Yes	
Treatment	0.3980	0.1433	ns	No	
ANOVA table	**SS**	**DF**	**MS**	**F (DFn, DFd)**	**P value**
Interaction	10.89	8	1.361	F (8, 161) = 1.894	P=0.0642
Cultivars	262.5	8	32.81	F (8, 161) = 45.67	P<0.0001
Treatment	1.554	1	1.554	F (1, 161) = 2.164	P=0.1433
Residual	115.7	161	0.7184		

* Alpha = 0.05; SS = sum of squares; DF= degrees of freedom; MS = mean squares; F (DFn, DFd) = F ratio (MS value divided by residual).

Supplementary Table 2. Shoot induction in calli of LSC and B36464 on varying concentrations of BAP after 4 weeks of culture.

Genotype	Media	Number of calli cultured	Mean number of shoots / callus
B36464	SIM1	16	9.69 ± 2.55^{a}
	SIM2	16	13.75 ± 4.31^{ac}
	SIM3	16	17.31 ± 7.90^{bc}
	SIM4	16	18.13 ± 3.07^{b}
LSC	SIM1	16	8.44 ± 4.19^{a}
	SIM2	16	12.44 ± 3.61^{ac}
	SIM3	16	16.38 ± 6.03^{bc}
	SIM4	16	16.75 ± 3.55^{b}

Mean shoot production is the ratio of the number of shoots formed to the number of calli cultured expressed as a percentage. Data represents mean ± SD. Values in a column followed by different letters are significantly different from each other at $p \leq 0.05$ (Sidak's multiple comparison test).

Vol. 21(5), pp.218-236, May 2022
DOI: 10.5897/AJB2021.17450
Article Number: 7A96C4569187
ISSN: 1684-5315

http://www.academicjournals.org/AJB

ACADEMIC JOURNALS
expand your knowledge

African Journal of Biotechnology

Full Length Research Paper

Characterization and antimicrobial activities of lactic acid bacteria isolated from selected Nigerian traditional fermented foods

Chinedu Godspower Ohaegbu[1], Anayochukwu Chibuike Ngene[1,2*], Unyime Inyang Asuquo[1], Oluwatoyin Debby Coulthard[2] and Ejikeme Nwachukwu[1]

[1]Department of Microbiology, College of Natural Sciences, Michael Okpara University of Agriculture Umudike, Abia State, Nigeria.
[2]Department of Microbiology, Faculty of Natural Sciences, University of Jos, Plateau State, Nigeria.

Received 31 December, 2021; Accepted 11 March, 2022

For production and preservation of traditional fermented foods, the genera, lactic acid bacteria (LAB) have been used. This study was carried out to determine the characteristics and the antimicrobial activities of lactic acid bacteria isolated from selected Nigerian traditional fermented foods. Changes in pH and titratable acidity (TA) of the samples were investigated for a period of four days (96 h). Eleven tentative LAB from fermented maize and cassava (Ogi and Fufu, respectively) were isolated and characterized. The spoilage organisms from fish were aseptically identified and the antimicrobial activity was determined by agar well diffusion method against three isolated food spoilage organisms (*Pseudomonas aeruginosa, Enterobacter aerogene* and *Bacillus cereus*). The isolates were selected and further identified as *Lactobacillus amylolyticus* strain L6, *Lactobacillus plantarum* strain ci-4w and *Lactobacillus sakei* strain MLS1 by the aide of genotypic characteristics (16S rRNA gene sequences). These strains were screened for their EPS producing activity, resistance to low pH and bile salts as well as bacteriocin activity. These strains can be used as starter culture or protective cultures to improve the hygiene, quality and increased safety of the food products by inhibiting the food borne pathogens and spoilage microorganisms.

Key words: Lactic acid bacteria, fermented foods, exopolysaccharides, antimicrobial activity, ogi, fufu.

INTRODUCTION

Over recent decades, lactic acid bacteria (LAB) have received much attention due to the health-promoting properties of certain strains, called probiotics. They are normal inhabitants of the healthy gut microbiota as they improve the balance of the microbial community in the intestine, confer protection against potential pathogenic bacteria, prevent or cure intestinal diseases and present in numerous fermented food products (Ngene et al., 2019; Rijkers et al., 2011; Brown and Valiere, 2004; Adak et al., 2002). LAB are used in a wide range of fermented food, they play a critical role in food processing and spontaneous fermentation (Elayaraja et al., 2014); also,

*Corresponding author. E-mail: ngene.anayochukwu@mouau.edu.ng.

they have been shown to be a major potential for use in biopreservation due to the fact that they are generally recognized as safe (GRAS) status (Salem, 2012; Vignolo et al., 2008; Radha and Padmavathi, 2015). They exert a strong antagonistic activity against many food contaminating microorganisms and these effects are mediated by production of antimicrobial metabolites such as organic acids (for example lactate, acetate, and butyrate), hydrogen peroxide, bacteriocins, and competition with harmful bacteria for nutrients or adhesion receptors (Maurya and Thakur, 2012; Wilson et al., 2011). LAB are among the most important microbes which are used in food fermentations, as well as in enhancing taste and texture in fermented food products (Van Geel-Schuttená et al., 1998; Hati et al., 2013).

One of the concerns in food industry is the contamination by food spoilage microorganisms and pathogens, which are frequent cause of food spoilage and food borne diseases. An important aspect of food contamination by microorganisms is the presence of potentially pathogenic species, which pose a great risk for the human and animal health (Broberg et al., 2007). Bacteria and various fungi are the cause of spoilage and can create serious consequences for the consumers and some troublesome spoilage microorganisms include aerobic psychrotrophic Gram-negative bacteria, yeasts, molds, heterofermentative lactobacilli, and spore-forming bacteria. They can cause extensive damage of the food such as unpleasant smell, taste or appearance as well as formation of harmful substances for the consumer's health (Dinev et al., 2017; Garcia et al., 2010; Garcha, 2018).

In order to achieve improved food safety against such spoilage microorganisms, food industry makes use of chemical preservatives or physical treatments (e.g. high temperatures). Many drawbacks which include the proven toxicity of the chemical preservatives has been recorded for these preservation techniques (e.g. nitrites), the alteration of the organoleptic and nutritional properties of foods, and especially recent consumer demands for safe but minimally processed products without additives (Ananou et al., 2007; Sharma et al., 2006).

However, the increasing resistance of food spoilage microorganisms to current preservatives, the consumer's high demand for safe, minimally processed foods, the alteration of the organoleptic and nutritional properties of foods and the hazards associated with the use of high doses of chemical preservatives have led to the need for finding safer alternatives in food preservation and disease prevention (Garcia et al., 2010; Nath et al., 2013).

Therefore, the need for alternatives to extend the shelf life of foods without changing their sensory properties and use in the treatment or prevention of gastrointestinal disease have launched research on probiotics and biopreservation technologies, which are based on the use of non-pathogenic microorganisms (lactic acid bacteria) or their metabolites to retard food spoilage or to improve food safety and confer health benefit (De Martinis et al., 2001; Ross et al., 2002). This research is designed to evaluate probiotics properties and to enhance the shelf-life of fermented food through assessing the biopreservation potency of lactic acid bacteria with the aim of developing starter/protective culture with predictable characteristics, for use in industrial application. The aim of this research is to analyze antimicrobial activities of exopolysaccharide lactic acid bacteria isolated from Ogi and Fufu on food spoilage organisms.

MATERIALS AND METHODS

Source and collection of samples

Maize grains (from two maize varieties: white and yellow) and freshly harvested cassava root tubers were purchased from a local market in Uyo, Akwa Ibom State, Nigeria. They were immediately processed and transported aseptically to the Post Graduate Microbiology laboratory, University of Uyo, for further analysis.

Sample preparation

The fermentation of maize grains and cassava tubers to produce Ogi and fufu, respectively, were carried out by simulating the traditional methods of processing (Figures 1 and 2). The fermentation of maize grains and cassava tubers to produce *ogi* and *fufu*, respectively, were carried out by simulating the traditional methods of processing.

Physicochemical analysis

Determination of pH

Samples of fermenting cassava mash (10 g) were homogenized in distilled water (100 ml) then the pH of the fermenting substrates was measured (Achi and Akomas, 2006), whereas pH of the fermentation liquor of cereal samples was determined directly (Anumudu et al., 2018; Nwachukwu and Ijeoma, 2010b) using a pH meter and the changes in pH of fermenting samples were monitored daily for 4 days (96 h).

Determination of titratable acidity (TA)

In order to determine the titratable acidity (TA) 10 ml of the samples were transferred to a 50 ml measuring flask and filled up to 50 ml with distilled water. After mixing, 10 ml of the diluted sample were titrated against 0.1 M sodium hydroxide (NaOH) using phenolphthalein as indicator until a pink colour appeared. Each ml of 0.1 M NaOH is equivalent to 90.08 mg of lactic acid (Nwachukwu and Ijeoma, 2010b).

TA of lactic acid (mg/ml) = (ml NaOH × M NaOH × M.E) / Volume of sample used

Where ml NaOH is the volume of NaOH used, M NaOH is the Molarity of NaOH used, M.E = Equivalent factor = 90.08 mg (Wakil and Ajayi, 2013; Nwachukwu and Ijeoma, 2010b).

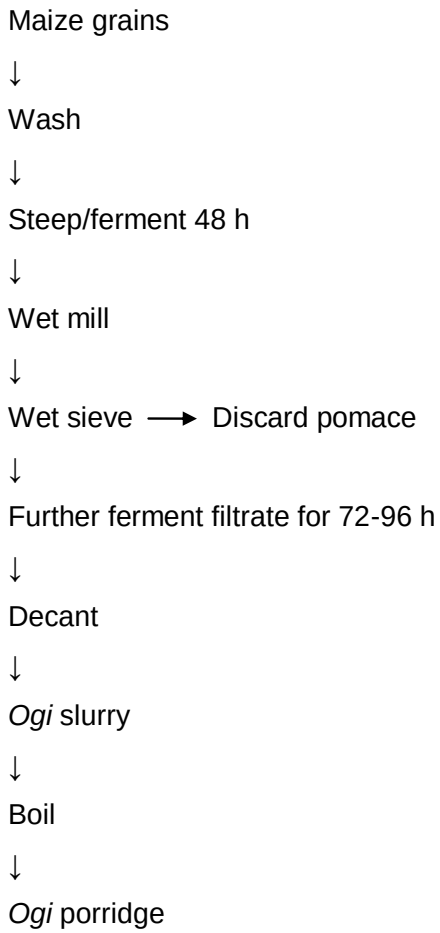

Figure 1. Flow chart for traditional method of *Ogi* processing.
Source: Hleba et al. (2021).

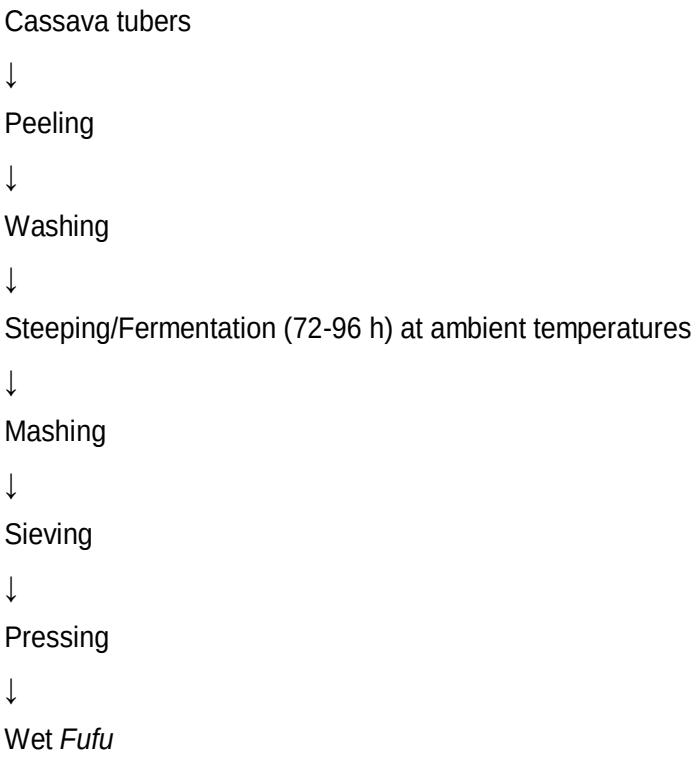

Figure 2. Flow chart for traditional method of *fufu* processing.
Source: Ayoade et al. (2018).

Microbiological analysis

Isolation and characterization of lactic acid bacteria

For preliminary identification, lactic acid bacteria (LAB) were isolated and enumerated using the De Mann, Rogosa and Sharpe (MRS) agar. A 1 ml of sample was diluted serially in ten folds dilution blanks properly mixed with sterile glass rod and 0.1 ml of diluted sample was introduced into sterile plate and molten sterile agar medium was poured (Harrigan and McCance, 1996; Ibeabuchi et al., 2014). The inoculated plates were incubated at 37°C for 48 h and suspected LAB colonies were picked randomly, sub-cultured on MRS agar to obtain pure culture and thereafter, pure cultures were grown on agar slants and kept at 4°C for further analysis.

LAB strains were characterized on the basis of their morphological, biochemical and physiological properties. Each isolate was examined under light microscope using oil immersion objectives after Gram-staining and Spore staining for the purpose of identification. All strains were subjected to Catalase test, Oxidase test, Motility test, Sugar fermentation (Bukola and Abiodun, 2008; Ibeabuchi et al., 2014), adjusted pH range of 4, 6, and 8, respectively using diluted buffer solutions, growth at different temperatures of 15, 25, 35, and 45°C and growth in the presence of different concentrations of NaCl (2, 4, 6 and 8%). After 24 to 48 h of incubation their growth were determined by observing their turbidity (Karthikeyan and Santosh, 2009). Probable identities were confirmed using Bergey's Manual of Systematic Bacteriology (Holt et al., 1994).

Isolation and identification of test organisms

The test organisms were isolated from Fish. A 10 g of the sample was aseptically cut and thoroughly blended with 10 ml sterile water using sterile blender. A 1 ml aliquot volume of the blended sample was measured out and homogenized in 9 ml of buffered peptone water and diluted serially in ten folds dilution blanks (Eze et al., 2011). 0.1 ml of the diluted sample was plated onto sterile Nutrient agar (Oxoid, England), Cetrimide agar and Eosin Methylene Blue (EMB) agar and incubated at 37°C under aerobic condition for 24 h The isolated bacterial colonies were identified on the basis of their morphological, cultural and biochemical characters and probable identities were confirmed using Bergey's Manual of Systematic Bacteriology (Holt et al., 1994).

Antimicrobial assay of lactic acid bacteria

In the screening of the LAB cells for antagonistic activity, the agar-well diffusion method was employed. Indicator lawns were prepared with 40 mL of Mueller Hinton Agar (MHA) seeded with 100 µL of an overnight culture of each food spoilage organism (*Pseudomonas aeruginosa, Enterobacter aerogene* and *Bacillus cereus*). With a sterile 5 mm diameter cork borer, wells were cut into the agar. Each LAB isolate were placed into each well. The plates were incubated at 30°C for 24 to 48 h after which they were examined for probable clearing of zones (Bali et al., 2011; Tambekar and Bhutada, 2010).

Evaluation of *in vitro* probiotic potentials of the selected lab isolates

Screening of the LAB isolates for exopolysaccharide production

Screening of exopolysaccharide from the LAB isolates was done using EPS selection medium (ESM 90 g of skimmed milk, 3.5 g of yeast extract, 3.5 g of peptone, 10 g of glucose/L) as described by van den Berg et al. (1993) and Patel et al. (2012). The isolates were inoculated differently into sterile ESM medium and incubated at 30°C for 24 h. The colonies developed thereafter were examined for mucoid and glistening by visual examination. The ropiness of the culture was also determined by touching the colonies with sterile wireloop and measuring the strings for extension of 5 mm or more.

Acid tolerance test

The acid resistance was performed by the viable plate count method (Hassanzadazar et al., 2012; Sahadeva et al., 2011). Bacterial cells were inoculated to adjusted pH of 2.0 and 3.0 and samples were taken every 3 h, thereafter, the viable colony counts were enumerated on MRS agar after incubation at 37°C for 6 h simulating the acidic environment in the human stomach.

Bile salt tolerance test

The bile tolerance test was carried out by method of Walker and Gilliland (1990). Briefly, cells of the selected strains were grown in MRS broth at 37°C overnight, and then subcultured in MRS broth containing different concentrations (0.1, 0.3, and 0.5%) of bile salts. The growth rate of each strain was determined by the viable plate count method after 24 h (Sahadeva et al., 2011).

Evaluation of biopreservation potential of lactic acid bacteria

Extraction of bacteriocins

Selected LAB isolates were grown in MRS broth at 37°C for 24 h. Cell free culture supernatant (CFCS) of each isolate was obtained by centrifugation at 3,000 rpm for 20 min. The supernatant was adjusted to pH 6.5 with 1M NaOH to neutralize any effect of acidity and inhibitory activity from hydrogen peroxide was eliminated by the addition of a 5 mg/ml catalase and subsequently filters sterilized through a 0.2 µm membrane filter (Onwuakor et al., 2014).

Detection of inhibitory activity of bacteriocin from selected isolates

The agar-well diffusion method was employed and 0.1 ml of test organisms (*P. aeruginosa, E. aerogene* and *B. cereus*) were plated using spread plate method on Mueller Hinton agar (MHA) plates. Wells were cut into the agar with a sterile 6 mm diameter cork borer. A 100 µL partially purified bacteriocins of each potential producer strain was placed into each well. The plates were then incubated at 30°C for 24 to 48 h after which they were examined for probable clearing zones (Bali et al., 2011).

Effect of pH on bacteriocin

Bacteriocin of the LAB isolates were adjusted to pH range of 4, 5, 6, 7, 8, and 9, respectively using diluted acid and base solutions and were allowed to stand at room temperature for 2 h. The residual bacteriocin activity of the cell free culture supernatant (CFCS) was then determined against the test organisms by well diffusion method and then measuring the diameter inhibition zone (Karthikeyan and Santosh, 2009).

Effect of temperature on crude bacteriocins

The isolated semi purified bacteriocins were subjected to different heat temperatures for 10 min at 40, 60, 80 and 100°C. Temperature stability was determined by measuring the residual antimicrobial activity of semi purified bacteriocins after treatments against the selected test organisms using the agar well diffusion assay (Karthikeyan and Santosh, 2009).

Effect of storage time on crude bacteriocin

The pH of cell free culture supernatant (CFCS) of the LAB isolates was adjusted and the effect of the organic acids and hydrogen peroxide (H_2O_2) was eliminated as stated earlier. 20 ml of the crude bacteriocin of LAB isolates was then stored at 37°C and 5ml of the crude bacteriocin was tested in well diffusion assay against the indicator organisms every 24 h for 4 days (96 h) using well diffusion method (Bali et al., 2011).

Molecular identification of lactic acid bacteria

DNA extraction

DNA extraction was conducted using the facilities of the Center for Molecular Biology and Biotechnology (CMBB), Michael Okpara University of Agriculture, Umudike (MOUAU), Nigeria. Genomic DNA from the isolated LAB was extracted with Zymo-Spin™ kits according to the manufacturer's instructions and the extracted DNA was separated on a 1% agarose gel electrophoresis.

PCR analysis

The 16S rRNA coding gene was amplified through polymerase chain reaction (PCR) using universal primers, 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 907R (5'-CCGTCAATTCMTTTRAGTTT-3'). PCR amplifications were carried out in a thermal cycler (T Gradient model, Biometera, Germany) using the following steps: one cycle of denaturation for 5 min at 94°C followed by thirty-five cycles of 94°C for 30 s. Annealing was performed at 50°C for 30 s, extension at 68°C for 1 min and final extension was done at 68°C for 10 min. The PCR products were kept at 4°C and the integrity of the PCR amplicons were separated on a 1% agarose gel electrophoresis (CSL-AG500, Cleaver Scientific Ltd) and visualized by staining with EZ-vision® Bluelight DNA Dye.

Sequencing

PCR products were purified using ExoSAP Protocol and sequencing was done with the Applied Biosystems™ BigDye™ Terminator v3.1 Cycle Sequencing Kit (Catalogue No. 4337455) using ABI 3500XL Genetic Analyser by Inqaba Biotec, South Africa.

BLAST analysis

The resulting 16S rRNA gene sequences were analyzed in NCBI

website (http://www.ncbi.nlm.nih.gov) using the Basic Local Alignment Search Tool (BLAST) Software in the GenBank nonredundant/nucleotide collection (nr/nt) to compare sequences and identification was performed on the basis of 16S rRNA sequence percentage similarity with the type strains.

Phylogenetic analysis

The sequences were aligned using ClustalW and the evolutionary history was inferred using the Neighbor-Joining method whereas the evolutionary distances were computed using the Maximum Composite Likelihood method. All ambiguous positions were removed for each sequence pair (pairwise deletion option) and the evolutionary analyses were conducted in MEGA X (Saitou and Nei, 1987; Kumar et al., 2018).

Statistical analysis

The experiments were carried out in replicate and results are given as the mean ± standard deviation. Data generated were subjected to One-Way analysis of variance (ANOVA), and Duncan Multiple range test was used for separation of mean, while $P<0.05$ was considered significant using Statistical Package for Social Sciences (SPSS) version 20.

RESULTS

Table 1 shows identification of indicator isolates (food spoilage organisms) from spoiled meat. The food spoilage organisms were identified based on morphological, cultural and biochemical characteristics as *P. aeruginosa, E. aerogenes*, and *B. cereus*.

The changes in the pH and titratable acidity during the spontaneous fermentation of *ogi* and *fufu* are presented in Figures 3 and 4. However, there was an increase in titratable acidity with a reduction in pH of the traditionally fermented food samples (*ogi* and *fufu*). There was a general decrease in pH from 6.32 to 5.02 (White *Ogi*), 6.20 to 4.58 (Yellow *Ogi*) and 6.43 to 4.29 (*Fufu*) after 96 h whereas titratable acidity increased from 0.35 to 0.70 (White *Ogi*), 0.30 to 0.60 (Yellow *Ogi*) and 0.15 to 0.60 after 96 h.

Isolation and identification of 11 LAB isolates were preliminarily isolated from traditionally fermented food samples (*Ogi* and *Fufu*) collected from a local market in Uyo, Akwa Ibom State and six LAB strains that showed a strong antibacterial activity against the indicator organisms were selected and preserved for further analysis. Based on the morphological, physiological and biochemical characteristics, these isolates were Gram-positive, rods (bacilli)-cocci, non-sporulating, non-motile, acid-tolerant, catalase and oxidase negative which grew in both aerobic and anaerobic environments (Tables 2 and 3).

The antimicrobial activity of all the tentative *Lactobacillus* strains had between 7.00 ± 0.00 and 22.00 ± 0.71 mm inhibitory zones towards the food spoilage organisms (Table 4). Six isolates designated as OG1, $W_2$2, $W_1$2, $Y_1$1, $F_1$2 and F6 showed a strong antibacterial activity against *P. aeruginosa, E. aerogene* and *B. cereus* ranging from 11.25±1.06 to 22.00±0.71 mm. The highest diameters of 22.00±0.71 and 20.20±0.42 mm were recorded for isolates coded $F_1$2, and OG1, while no activity was recorded for OG6.

These isolates were identified on the basis of genotypic characteristics (16S rRNA gene sequences similarity with the type strains) during BLAST searches as *Lactobacillus amylolyticus* strain L6, *Lactobacillus plantarum* strain ci-4w and *Lactobacillus sakei* strain MLS1 (Table 5).

Table 6 shows the exopolysaccharide (EPS) production of different LAB isolates and the results revealed that *L. amylolyticus* strain L6 showed the highest ropy strand formation.

In Table 7, the result showed that these *Lactobacillus* strains tolerated pH 2 and 3 of the medium for 3 and 6 h of incubation. Generally, there is a reduction in probiotic count, as they were exposed to pH 2 after 6 h with lower count 15.00±2.83 to 25.00±1.41 CFU/ml compared similarly at pH 3.0 after 6 h which recorded higher count 67.00±1.41 to 90.00±4.24 CFU/ml. The survival rate of these *Lactobacillus* strains supplemented with bile shows gradual decline in viable count as the bile concentrations increased (Figure 5).

The antimicrobial activity of crude bacteriocin from different LAB-producing strains as depicted in Table 8 showed that bacteriocins produced by *L. amylolyticus* strain L6, *L. plantarum* strain ci-4w and *L. sakei* strain MLS1 have broad spectrum activity against Gram positive (*Bacillus cereus*) and Gram negative bacteria (*P. aeruginosa* and *E. aerogene*). It produced high inhibition zone diameters ranging from 13.25±0.92 to 19.75±0.35 mm against the food spoilage organisms.

The effect of varied pH on inhibitory effects of bacteriocin indicates that bacteriocin activity was affected as different inhibition zones were observed among the various *Lactobacillus* strains against the indicator organisms (Figures 6 to 8). The highest antimicrobial productions were recorded mostly between pH 6.0 and 7.0, and inactivated at pH values above 8.0. The rate of antimicrobial production was indicated by increase in zones of inhibition (in diameter) from pH 5.0 to pH 7.0 followed by gradual decrease between pH 8.0 and 9.0.

Table 9 shows the effect of temperature on the bacteriocin activity against *P. aeruginosa, E. aerogenes* and *B. cereus*. Among the bacteriocins produced by the isolated Lactobacilli, however, bacteriocin produced by *L. sakei* strain MLS1 showed thermostability over a wide range of temperature from 40 to 80°C for 10 min. Generally, as the temperature increases, the decreases of antibacterial activity of the crude bacteriocins occur. Result showed that the highest significant ($P < 0.05$) zone of inhibition was recorded against *B. cereus* (19.25±0.35^a mm) at 40°C for 10 min.

The effect of varied storage time (h) on bacteriocin

Table 1. Morphological and biochemical characterization and identification of test organisms

Isolation code	Colour	Gram stain	Cell shape	Catalase	Indole	Citrate	Oxidase	Spore formation	Voges Proskauer	Motility	Urease	Methyl Red	Glucose	Lactose	Maltose	Probable organisms
A	Greenish on Cetrimide agar	-	Rod	+	-	+	+	-	-	+	-	-	-	-	-	*P. aeruginosa*
B	Pink to Purple on EMB agar	-	Rod	+	-	+	-	-	+	+	-	-	+	+	+	*E. aerogenes*
C	Blue-black with green metallic sheen on EMB agar	-	Rod	+	+	-	-	-	-	+	-	+	+	+	-	*E. coli*
D	Cream to off-white on Nutrient agar	+	Rod	+	-	+	-	+	+	+	-	-	+	-	+	*B. cereus*
E	Greyish white on Nutrient agar	-	Rod	+	-	-	-	-	-	+	-	-	+	-	+	*Salmonella* spp.
F	Cream to light yellow on Nutrient agar	+	Rod	+	-	+	+	+	+	+	-	-	+	+	+	*Bacillus* spp.

Table 2. Morphological and biochemical characterization and identification of lactic acid bacteria isolates.

Isolation code	Gram stain	Cell shape	Cell arrangement	Catalase	Oxidase	Spore formation	Motility	Acid/Gas production from glucose	Acid/Gas production from lactose	Acid/Gas production from maltose	Organism
OG1	+	Rods	Clusters	-	-	-	-	+/W+	+/+	+/+	*Lactobacillus amylolyticus* strain L6
$Y_1$1	+	Long rods	Chains	-	-	-	-	+/-	W+/-	+ /-	*Lactobacillus plantarum* strain ci-4w
$F_1$2	+	Rods	Pairs	-	-	-	-	+/+	W+/-	+/+	*Lactobacillus sakei* strain MLS1

+ Positive; - Negative; W+ Weakly positive.

activity was noted as different zones of inhibition were observed among the various *Lactobacillus* strains against the indicator organisms (Table 10). The highest inhibitory effects were recorded at 24 and 48 h storage period. The rate of antimicrobial production was indicated by increase in diameter of zones of inhibition from 24 to 48 h followed by gradual decrease between the storage time of 72 and 96 h.

DISCUSSION

Based on preliminary data obtained in this study, a total of 11 LAB strains isolated from different fermented food samples (white *Ogi*, yellow *Ogi* and *fufu*) were screened for antimicrobial activity against three indicator organisms (*P. aeruginosa, E. aerogenes* and *B. cereus*). Out of these LAB isolates (n=11), six LAB isolates exhibited good antimicrobial activity with the highest significant ($p < 0.05$); they were selected on the basis of their antimicrobial activities against indicator strains and were designated as $W_2$2 , $W_1$2, $Y_1$1, OG1, $F_1$2 and F6 based on the source of their isolation with OG1 and $F_1$2 having the highest inhibitory diameter of 18.25±0.35 and 22.00±0.71 mm against *Bacillus cereus,* 20.20±0.42 and 18.75±0.35 mm against *P. aeruginosa,* and 12.50±0.71 and 15.25±1.77 mm against *E. aerogene*, respectively (Table 4). Values obtained for this test coincide for some strains with the work of Amara et al. (2019), where *E. aerogenes* and *Citrobacter freundii* strains were inhibited by all LAB with inhibition zones ranging between 13 and 21 mm in diameter and antagonism of Lactobacilli was also observed on *B. cereus, Staphylococcus aureus* ATCC 433005*, Enterobacter cloacae, Escherichia coli* ATCC 25922*, P. aeruginosa* ATCC 27853 and *Klebsiella pneumonia*. Due to several antagonistic characteristics of LAB which include: bacteriocins or hydrogen peroxide production, lowering of pH by production of organic acids in the medium, and nutrients competition, inhibits the spoilage organisms/pathogens (Bezkorvainy, 2001; Isolauri et al., 2004; Charlier et al., 2009; Merzoug et al., 2016, 2018). The maximum antibacterial potential shown by these *lactobacillus* strains against the food spoilage bacteria indicated that these strains could be used as probiotics and biopreservatives for extending the storage life and quality of food.

The LAB isolates were characterized and identified on the basis of morphological, physiological, biochemical and genotypic characteristics (16S rRNA gene sequences similarity with the type strains) (Tables 2, 3 and 5).

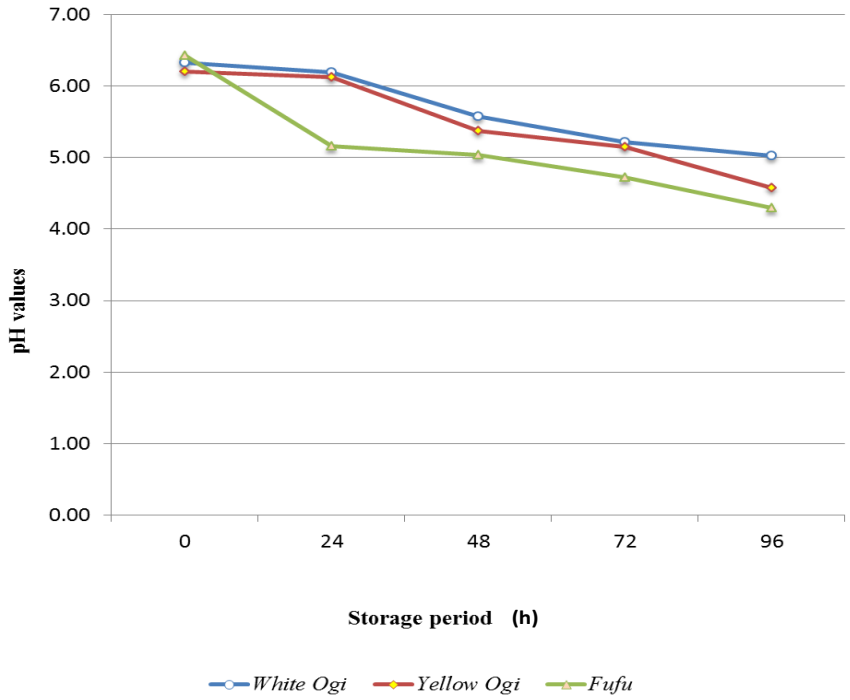

Figure 3. Changes in pH of fermented food products (*Ogi* and *Fufu*).

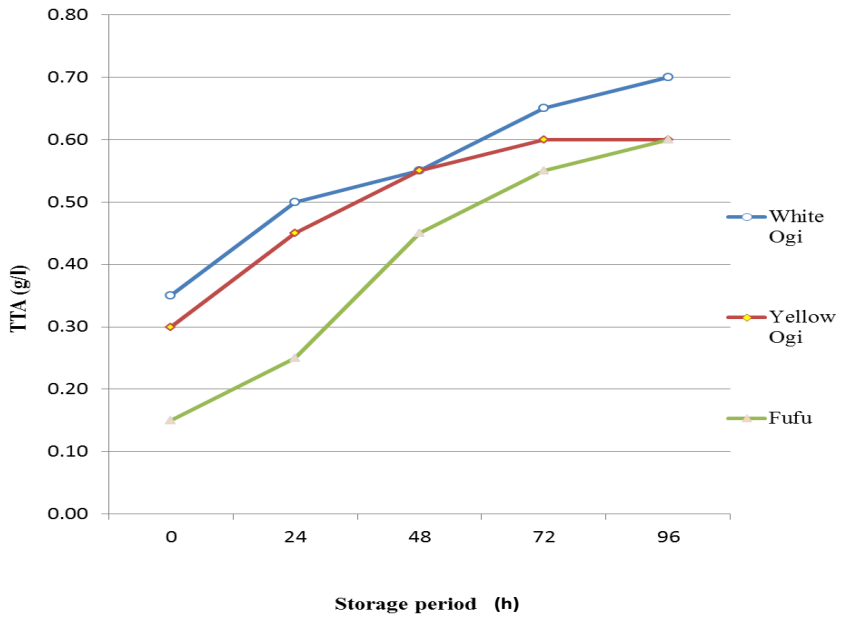

Figure 4. Changes in titratable acidity (TA) of LAB isolates during 96 h growth.

The identification carried out for representative Lactobacillus species from the traditional fermented food products (ogi and fufu) demonstrated the dominance of L. amylolyticus strain L6, L. plantarum strain ci-4w and L. sakei strain MLS1. Lactobacillus species have been reported to be predominant in fermented foods (Ogunshe et al., 2007). Nwokoro and Chukwu (2012) reported that the microbial composition of Akamu sample within the first 48 h of fermentation included Lactobacillus delbrueckii, L. plantarum, Lactobacillus fermentum and Lactobacillus amylovorus.

Changes in pH and titratable acidity during the ogi and fufu fermentation showed a marginal increase in titratable acidity with reduction in pH values (Figures 3 and 4).

Table 3. Physiological characterization of isolates.

Organism	NaCl (%)			Growth rate							
				Temperature (°C)				NaCl (%)			
	pH 4	pH 6	pH 8	15°C	25°C	35°C	45°C	(2%)	(4%)	(6%)	(8%)
Lactobacillus amylolyticus strain L6	+++	+++	+++	-	+	+++	+	+++	+++	+	+
Lactobacillus plantarum strain ci-4w	+	+++	+++	+	+++	+++	-	+++	+++	+++	+
Lactobacillus sakei strain MLS1	+++	+++	+++	-	+++	+++	-	+++	+++	+	+

+++ Luxurious growth; + growth; - no growth.

Table 4. Inhibitory activity of the LAB isolates against different test organisms.

Isolate code	Source	Inhibition profile (mm)		
		Indicator organisms		
		Pseudomonas aeruginosa	*Enterobacter aerogene*	*Bacillus cereus*
OG1	White *Ogi*	20.20 ± 0.42^{a}	12.50 ± 0.71^{c}	18.25 ± 0.35^{b}
OG6		0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
$W_2$2		14.75 ± 1.06^{b}	18.50 ± 0.00^{a}	12.45 ± 0.78^{b}
$W_1$2		18.75 ± 0.35^{a}	16.45 ± 2.05^{ab}	11.50 ± 2.12^{b}
$Y_1$1	Yellow *Ogi*	13.55 ± 1.34^{c}	16.50 ± 0.71^{b}	19.50 ± 0.00^{a}
$Y_2$2		12.25 ± 0.35^{a}	0.00 ± 0.00^{c}	8.00 ± 0.71^{b}
$F_2$1	*Fufu*	12.00 ± 1.41^{b}	9.00 ± 0.71^{b}	15.75 ± 1.06^{a}
$F_1$2		18.75 ± 0.35^{ab}	15.25 ± 1.77^{b}	22.00 ± 0.71^{a}
F4		14.75 ± 1.06^{a}	9.25 ± 1.06^{b}	12.50 ± 0.71^{a}
F5		11.90 ± 0.85^{a}	0.00 ± 0.00^{c}	8.50 ± 0.71^{b}
F6		20.00 ± 0.00^{a}	11.25 ± 1.06^{c}	16.95 ± 0.07^{b}

Values are presented as means ± standard deviation and mean values in the same row having different superscript letters (a,b,c) are significantly different ($p<0.05$)

However, fermentation caused a general decrease in pH from 6.32 to 5.02 (White Ogi), 6.20 to 4.58 (Yellow Ogi) and 6.43 to 4.29 (Fufu) after 96 h whereas titratable acidity increased from 0.35 to 0.70 (White Ogi), 0.30 to 0.60 (Yellow Ogi) and 0.15 to 0.60 during 96 h fermentation. The reduction of the pH values towards acidity was possibly due to fermentation by the LAB (Abegaz, 2007). According to Inyang and Idoko (2006), an increase in acidity as the fermentation progressed was because of the accelerated growth rate of LAB. The amount of acid produced during fermentation increased exponentially with decrease in pH is in agreement with the findings of Nwachukwu and Ijeoma (2010b) which indicated an increase in titratable acidity with a reduction in pH during fermentation of maize for *Ogi*. Also, Nwokoro and Chukwu (2012) observed pH decreased from 6.6 at the start of the Akamu fermentation to 3.9 after 72 h while titratable acidity increased from 0.48 at 0 h to 0.79 after 72 h. During the steeping of maize grains for *Ogi* production, the activities of lactic acid bacteria which is responsible for the production of lactic acid may result to decrease in pH (Odunfa and Adeyele, 2000). Whereas an increase in titratable acidity with declined pH during 96 h of fufu fermentation was in accordance with Achi and Akomas (2006) who demonstrated that the initial pH of retted cassava samples was 6.8, followed by subsequent decrease to 3.8 at the end of 96 h fermentation. Also, Oyedeji et al. (2013) observed that the pH of the fermenting cassava roots decreased from 5.6 to 3.7 during 72 h fermenting period while the total titratable acidity (in % lactic acid) increased from 0.07 ± 0.01 to 0.21 ± 0.01 and decreased to 0.09 by the end of 72 h fermentation period whereas the pH of fermenting maize grains dropped from 5.9 to 3.8 by the end of the 72 h fermentation period while the total titratable acidity increased from 0.13 ± 0.01 to 0.28 ± 0.01 after 24 h and then decreased to 0.14 by the end of fermentation period which is significant to this present study which confirms that there is a reduction in the pH with increase in the titratable acidity (production of organic acids) during *ogi* and *fufu* fermentation.

The screening for exopolysaccharide (EPS) producing

Table 5. Molecular identification of LAB isolates.

Isolate code	Nucleotide sequence	(%) Similarity	16S rRNA analysis result	Accession number
OG1	GGGRGGCTACACATGCAAGTCGAGCGGTAACAGGAGAA AGCTTGCTTTCTTGCTGACGAGCGGCGGACGGGTGAGT AATGTATGGGGATCTGCCCGATAGAGGGGGATAACTAC TGGAAACGGTGGCTAATACCGCATAATGTCTACGGACCA AAGCAGGGGCTCTTCGGACCTTGCACTATCGGATGAAC CCATATGGGATTAGCTAGTAGGTGGGGTAAAGGCTCAC CTAGGCGACGATCTCTAGCTGGTCTGAGAGGATGATCA GCCACACTGGGACTGAGACACGGCCCAGACTCCTACGG GAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGC CTGATGCAGCCATGCCGCGTGTATGAAGAAGGCCTTAG GGTTGTAAAGTACTTTCAGCGGGGAGGAAGGTGATAAG GTTAATACCCTTRTCAATTGACGTTACCCGCAGAAGAAG CACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGG AGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGC GCACGCAGGCGGTCAATTAAGTCAGATGTGAAAGCCCC GAGCTTAACTTGGGAATTGCATCTGAAACTGGTTGGCTA GAGTCTTGTAGAGGGGGGTAGAATTCCATGTGTAGCGG TGAAATGCGTAGAGATGTGGAGGAATACCGGTGGCGAA GGCGGCCCCCTGGACAAAGACTGACGCTCAGGTGCGAA AGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTC CACGCTGTAAACGATGTCGATTTAGAGGTTGTGGTCTTG AACCGTGGCTTCTGGAGCTAACGCGTTAAATCGACCCG CCTGGGGAGTACGGCGCAAGGTTAAAACTCAAATGAATT TGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTAA TTTCGATGCACGCGGAGRACCTTACCTACTCTTGACATC CAGCGAATCCTTAAGARAWGGAGGAGTGGCTTCCGGAC GCTGARGACAGTGCTGCATGCTGCCGTCAGCTCCTGTT TGGAATGACGCTAAAGTCCCGCAACAGASTGGCA	80	*Lactobacillus amylolyticus* strain L6	CP020457.1
$Y_1$1	CGGSGGGCTAATACATGCAGTCGAGCGAACAGAKAAGG AGCTTGCTCCTTTGACGTTAGCGGCGGACGGGTGAGTA ACACGTGGGCAACCTRCCTTWTAGWTTGGGATAACTCC GGGAAACCGGGGCTAATACCGAATAATCTGTTTCACCTC ATGGTGAAATATTGAAAGACGGTTTCGGCTGTCGCTATW GGATGGGCCCGCGGCGCATTAGCTAGTTGGTGAGGTAA CGGCTCACCAAGGCGACGATGCGTAGCCGACCTGAGA GGGTGATCGGCCACACTGGGACTGAGACACGGCCCAG ACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCACAAT GGGCGAAAGCCTGATGGAGCAACGCCGCGTGAGTGAA GAAGGATTTCGGTTCGTAAAACTCTGTTGTAAGGGAAGA ACAAGTACAGTAGTAACTGGCTGTACCTTGACGGTACCT TATTAGAAAGCCACGGCTAACTACSTGCCARCAGCCGC GGTAATACSTARGTGGCAAGCGTTGTCCGGAATTATTGG GCGTAAAGCGCGCGCAGGTGGTTTCTTAAGTCTGATGT GAAAGCCCACGGCTCAACCGTGGAGGGTCATTGGAAAC TGGGAGACTTGAGTGCAGAAGARGATAGTGGAATTCCA AGTGTAGCGGTGAAATGCGTAGAGATTTGGAGGAACAC CAGTGGCGAAGGCGACTATCTGGTCTGTAACTGACACT GAGGCGCGAAAGCGTGGGGAGCAAACAGGATTAGATAC CCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTT AGGGGTTTCCGCCCCTTTAGTGCTGCAGCTAACGCATTA AGCACTCCGCCTGGGGAGTACGTCGCAAGACTGAAACT CAAGGAATTGACGGGGCCGCACAGCGGTGGAGCATTGT GGTTAATTCGAAGCACGCGAGACCTTACCAGTCTTGACA TTCCGTTGACACTTGTAARGATWTTGGTTTCCCTTTCGR GCACGTGACMGGKGTGCATGGTTGYCTCACTCGTGTCK GGRATGTTGGGTTAGTCCGACGAGGCCAACTTGAATYTT AGTTGCATTCATTTATTTGGAC	90	*Lactobacillus Plantarum* strain ci-4w	MF480392.1

Table 5. Contd

$F_1$2	GGSSGTCTATACATGCAAGTCGAGCGAACAGAGAAGGA GCTTGCTCCTTTGACGTTAGCGGCGGACGGGTGAGTAA CACGTGGGCAACCTACCTTATAGTTTGGGATAACTCCGG GAAACCGGGGCTAATACCGAATAATCTATTTCACTTCATG GTGAAATACTGAAAGACGGTTTCGGCTGTCGCTATAAGA TGGGCCCGCGGCGCATTAGCTAGTTGGTGAGGTAACGG CTCACCAAGGCGACGATGCGTAGCCGACCTGAGAGGGT GATCGGCCACACTGGGACTGAGACACGGCCCAGACTCC TACGGGAGGCAGCAGTAGGGAATCTTCCACAATGGGCG AAAGCCTGATGGAGCAACGCCGCGTGAGTGAAGAAGGT TTTCGGATCGTAAAACTCTGTTGTAAGGGAAGAACAAGT ACAGTAGTAACTGGCTGTACCTTGACGGTACCTTATTAG AAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAAT ACGTAGGTGGCAAGCGTTGTCCGGAATTATTGGGCGTAA AGCGCGCGCAGGCGGTCCTTTAAGTCTGATGTGAAAGC CCACGGCTCAACCGTGGAGGGTCATTGGAAACTGGGGG ACTTGAGTGCAGAAGAGGAAAGTGGAATTCCAAGTGTAG CGGTGAAATGCGTAGAGATTTGGAGGAACACCAGTGGC GAAGGCGACTTTCTGGTCTGTAACTGACGCTGAGGCGC GAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTA GTCCACGCCGTAACGATGAGTGCTAAGTGTTAGGGGGT TTCCGCCCCTTAGTGCTGCAGCTACGCATTAAGCMCTCC GCCTGGGGAGTACGGTCGCAAGACTGGAAACTCAAGGG AATTTGACGGGGGTCCCGCAMAAGCGGTGAGCATGTGT TAATTCGAATSCAACGCGAAGAACTTACAGCCTTGGACA TCCGTTGACMCTGKAAARATATGGGTWTCCATTCCGGAC AACGKACAGTGGCCATGGTTGCGTCAGTCGGTCCTGAAT GTGGGTAAGTCCGCGAGACCARC	99	*Lactobacillus sakei* strain MLS1	MG654786.1

Table 6. Exopolysaccharide (EPS) production by different LAB isolates

Organism	EPS productivity		
	Glistening	Slimy (Mucoid)	Ropiness (mm)
Lactobacillus amylolyticus strain L6	+	+	6.50±1.41[a]
Lactobacillus plantarum strain ci-4w	+	+	4.75±1.06[ab]
Lactobacillus sakei strain MLS1	+	+	1.85±0.07[b]

Values are presented as means ± standard deviation and mean values in the same column having different superscript letters (a,b,c) are significantly different ($p<0.05$)

Lactobacilli revealed that all studied lactic acid bacteria strains (n=3), showed slimy and ropy colonies, however, *Lactobacillus amylolyticus* strain L6 isolated from white Ogi showed highly ropy strand formation of 6.50±1.41 mm followed by *L. plantarum* strain ci-4w isolated from yellow Ogi with 4.75±1.06 mm (Table 6) and this is in conformity with the results of Mostefaoui et al. (2014) which revealed the presence of twelve (12) mucoid and ropy strains of *Lactobacillus* of twenty six (26) tested strains in culture collection. This finding is also similar to the investigation of Adebayo-Tayo and Onilude (2008) which showed that more than 64.29% of the studied *L. plantarum* strains were active producers of exopolysaccharide, also, the EPS produced by *L. plantarum* (LPWO11) strain isolated from "white Ogi" and *L. casei ssp tolerans* (LCN6) isolated from "fufu" had the highest while the strain isolated from "brown Ogi" (LPBOI4) had the lowest EPS activity. Moreover, Mostefaoui et al., (2014) has reported that microbial EPS are not consumed as an energy source by the producing microorganism, but are released to protect the producer organism during starvation conditions and also at extreme pH and temperature conditions (Mostefaoui et al., 2014). Therefore, exopolysaccharide production is considered an important probiotic attribute of lactic acid bacteria in the present search for human probiotic LAB.

Similarly, other important criteria to be a good source of probiotics are the tolerance to bile and high acid levels, which is present in the small intestine of the stomach (Dunne et al., 2001; Jena et al., 2013; Garcia et al.,

Table 7. Survival of probiotic LAB at acidic pH levels**.**

Bacteriocin-producing LAB strains	Growth rate (Log Cfu/ml)			
	Acid tolerance			
	3 h		6 h	
	pH 2.0	pH 3.0	pH 2.0	pH 3.0
Lactobacillus amylolyticus strain L6	16.00 ±1.41^{c}	61.50±4.95^{b}	20.00±1.41^{c}	90.00±4.24^{a}
Lactobacillus plantarum strain ci-4w	27.50±3.54^{c}	52.50±6.36^{b}	15.00±2.83^{d}	67.00±1.41^{a}
Lactobacillus sakei strain MLS1	35.00±1.41^{c}	66.50±3.54^{b}	25.00±1.41^{d}	83.00±5.66^{a}

Values are presented as means ± standard deviation and mean values in the same row having different superscript letters (a,b,c) are significantly different ($p<0.05$).

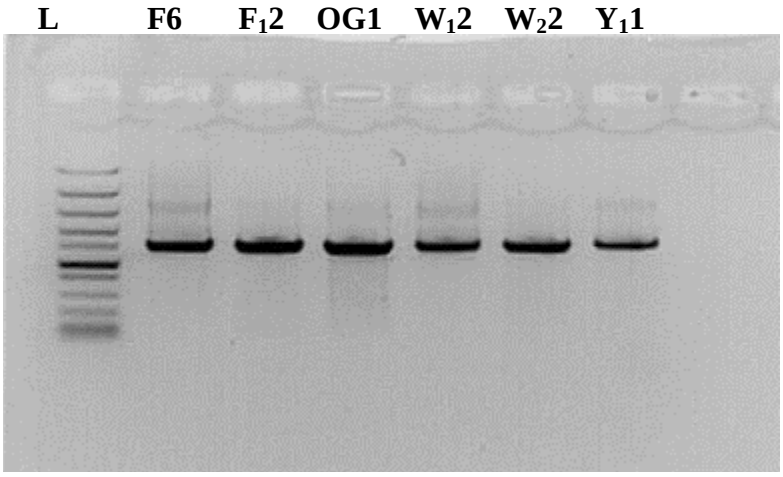

Figure 5. Agarose gel electrophoresis of the 16S rRNA gene of the *Lactobacillus amylolyticus* strain L6 (OG1), *Lactobacillus Plantarum* strain ci-4w ($Y_1$1) and *Lactobacillus sakei* strain MLS1 ($F_1$2). Lane L represents the 100 bp molecular ladder.

2016). The three LAB strains, *L. amylolyticus* strain L6, *L. plantarum* strain ci-4w and *L. sakei* strain MLS1 showed acid tolerance at pH 2 and pH 3 (Table 7), and high bile salt tolerance at 0.3 and 0.5% (Figure 7) which is considered a prerequisite for colonization and metabolic activity of these LAB strains in the small intestine of the host. As cited by Sultana et al. (2000) and Chan and Zhang (2005), low pH environments are thought to inhibit the metabolism activity and growth of LAB, thus reducing the probiotics' viability as aciduric members of LAB, as these cells were proven to be vulnerable at pH 2.0 and below, where *L. acidophilus* could not survive in acidic pH environment. In this present study, *L. amylolyticus* strain L6, *L. plantarum* strain ci-4w and *L. sakei* strain MLS1 growth decreased with increasing duration of 6 h at pH 2 and remained constantly stable at higher pH 3 for 6 h which is in accordance with the study conducted by Mandal et al. (2006). This is also in agreement with the findings of Fernandez et al. (2003) who reported that good probiotic sources should withstand at least pH range of 3.0 but it contrasts the findings of Sahadeva et al. (2011) who reported that there was a reduction in probiotic count, as they were exposed to pH 1.5 and 3.0 and the count was fairly constant at pH 7.2 (control). The present finding indicates that there is no correlation between pH 2 and 3 during the growth period (h) of LAB strains because as viable count at pH 2 was decreasing, viable count at pH 3 was increasing.

The isolated probiotic strains proved to exhibit an excellent quality of bile tolerance. Data obtained from the acid tolerance study indicated that LAB strains that survive at high acidic pH 2 were also able to grow in the presence of the subsequent bile salt test. The findings in this study are in accordance to Leyer and Johnson (1993) who postulated that acid and bile have separate and combined effects on the growth of bacteria as bile stress takes place after pH stress in the stomach. The enhanced survival capabilities appeared to be due to the adaptation ability or acclimatization of the bacteria to the low pH environment, therefore minimizing the relative

Table 8. Inhibitory effect of crude bacteriocin

Bacteriocin-producing LAB strains	Inhibition Profile (mm)		
	Indicator Organisms		
	Pseudomonas aeruginosa	***Enterobacter aerogene***	***Bacillus cereus***
Lactobacillus amylolyticus strain L6	13.25±0.92[b]	14.75±1.06[b]	18.50±0.00[a]
Lactobacillus plantarum strain ci-4w	14.00±0.71[b]	15.75±1.06[ab]	18.25±1.06[a]
Lactobacillus sakei strain MLS1	19.75±0.35[a]	16.45±0.07[c]	18.95±0.07[b]

Values are presented as means ± standard deviation and mean values in the same row having different superscript letters (a,b,c) are significantly different ($p<0.05$).

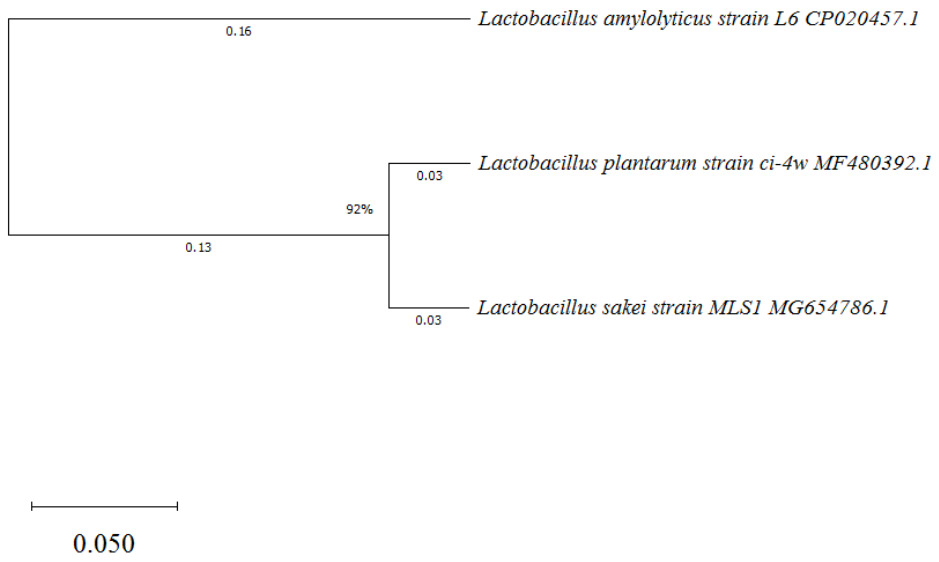

Figure 6. Evolutionary relationship of the isolated LAB strains.

toxicity to glycoconjugates in the intestine (Begley et al., 2005; Martoni et al., 2007). Therefore, the results of the present study on acid and bile tolerance exhibit that these. Lactobacilli will reach the small intestine and colon and thus contribute in balancing the intestinal microflora. However, studies concerning bacteriocins produced by LAB have received an increasing interest because of the potential use of bacteriocins as food preservatives and bacteriocins are supposed to act only on closely related species, which limits their application as a good preservative (Cleveland et al., 2001). In contrast, this study revealed that bacteriocins produced by *L. amylolyticus* strain L6, *L. plantarum* strain ci-4w and *L sakei* strain MLS1 have broad spectrum activity against Gram positive (*B. cereus*) and Gram negative bacteria (*P aeruginosa* and *E aerogenes*). It showed a good antagonistic activity against these potent food spoilage organisms, which shows its efficacy and potential application as a natural food preservative. Adesokan et al. (2009) recorded a similar result against *P. aeruginosa, S . aureus*, and *E. coli*. Todorov et al. (2012) also obtained similar results in the case of bacteriocin produced by *L. sakei* ST22Ch which inhibited the growth of *Pseudomonas* and *Staphylococcus* species. However, several investigation revealed that *L. plantarum* strains produce a broad range of bacteriocins such as ST28MS, ST26MS, bacST202Ch, bacST216Ch, ST71KS, AMA-K, plantaricin B, D, G, K, etc., (Enan et al., 1996; Todorov and Dicks, 2005; Hata et al., 2010; Gong et al., 2010; Martinez et al., 2013). According to the report by Dinev et al. (2017), *L. plantarum* exerts inhibitory activity against a variety of potentially harmful Gram-positive bacteria including *Listeria monocytogenes*, *S. aureus* and some members of the genera *Bacillus*, *Clostridium*, and *Enterococcus* as well as being active against many Gram-negative pathogens and food spoilage microorganisms including *Escherichia coli* (including enteropathogenic, enterotoxigaenic, enteroinvasive, multidrug-resistant enteroaggregative *E. coli* and *E. coli* 0157:H7), *P. aeruginosa*, *Yersinia enterocolitica*, *Campylobacter jejuni*, *Helicobacter pylori*, *Klebsiella*, *Salmonella*, and *Shigella* species which is in conformity

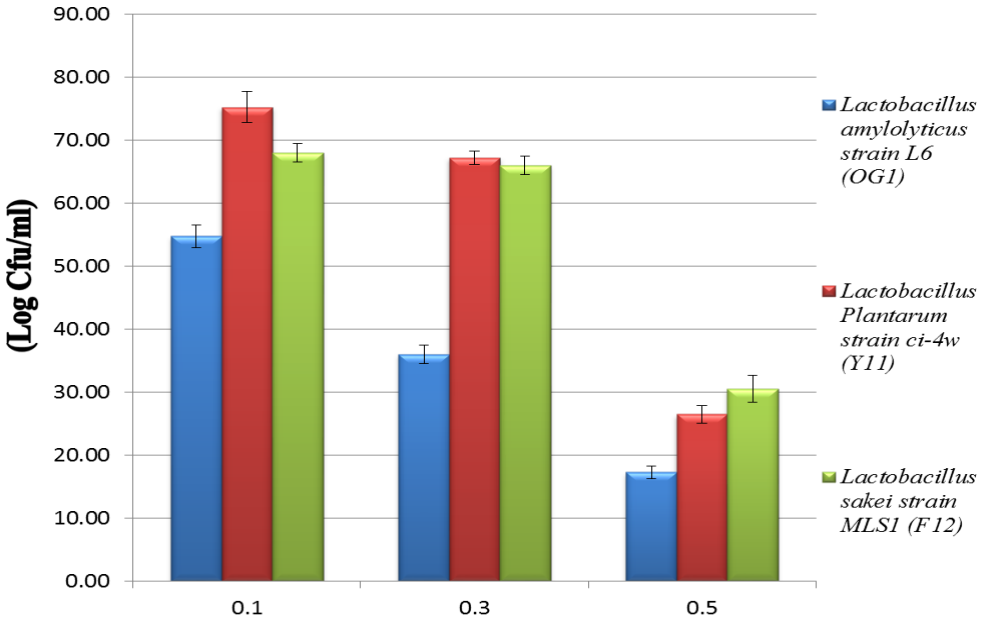

Figure 7. Growth of lactic acid bacteria at different bile salt.

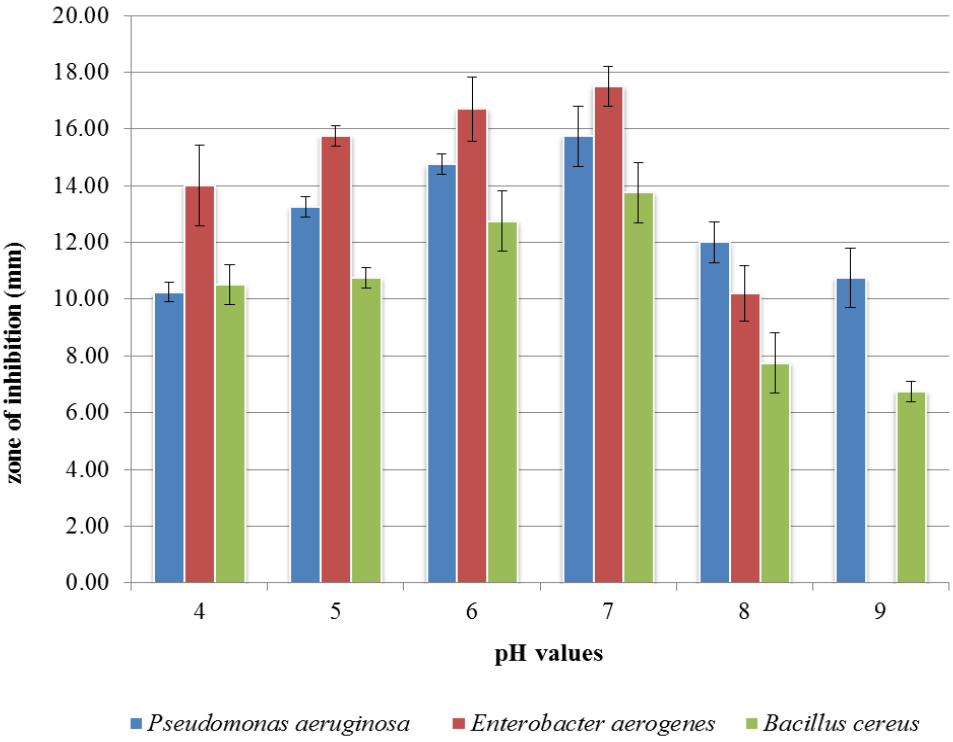

Figure 8. Effect of pH on crude bacteriocin activity of *Lactobacillus amylolyticus* strain L6.

with the present study. Report by Ogunshe et al. (2007) reported that *Lactobacillus* strains produced a bacteriocin compounds which can inhibit several bacteria. Lactic acid bacteria have potentials to inhibit the growth of pathogenic and food spoilage bacteria and the possibilities exist for their use as probiotic LAB (Obi, 2018). Therefore, possession of bacteriocin in Lactobacilli reveals their probiotic and biopreservation potentials.

Bacteriocin production as well as its activity seemed to be influenced by some factors such as the pH of culture

Table 9. Stability of crude bacteriocin to temperature.

Bacteriocin-producing LAB strains	Test organisms	Temperature (°C)			
		40	60	80	100
L. amylolyticus strain L6	*P. aeruginosa*	12.25±0.35^{a}	11.50±0.71^{b}	0.00±0.00^{a}	ND
	E. aerogene	12.00±0.71^{a}	0.00±0.00^{a}	0.00±0.00^{a}	ND
	B. cereus	17.75±0.35 ^{b}	13.50±1.41^{b}	9.75±1.06^{b}	ND
L. plantarum strain ci-4w	*P. aeruginosa*	13.50±0.71^{a}	9.75±0.35^{a}	0.00±0.00^{a}	ND
	E. aerogene	14.75±0.35^{ab}	11.00±0.71^{a}	7.00±0.00^{b}	ND
	B. cereus	16.50±0.71^{b}	12.75±0.35^{b}	7.75±0.35^{c}	ND
L. sakei strain MLS1	*P. aeruginosa*	17.25±0.35^{b}	14.50±0.00^{ab}	9.25±1.77^{a}	ND
	E. aerogene	14.25±0.35^{a}	11.75±1.06^{a}	7.25±0.35^{a}	ND
	B. cereus	19.25±0.35^{c}	15.25±1.06^{b}	10.25±0.35^{a}	ND

Values are presented as means ± standard deviation and mean values in the same row having different superscript letters (a,b,c) are significantly different (p<0.05). ND: Not detected.

Table 10. Effect of Storage time on bacteriocin activity.

Bacteriocin-producing LAB strains	Test organisms	Storage time (h)					
		0	24	48	72	96	120
L. amylolyticus strain L6	*P. aeruginosa*	14.35±0.92^{a}	12.75±0.35^{a}	9.75±1.06^{a}	7.50±0.00^{b}	0.00±0.00	0.00±0.00
	*E. aerogene*s	14.75±1.06^{a}	13.00±1.41^{a}	15.15±0.92^{b}	0.00±.000^{a}	0.00±0.00	0.00±0.00
	B. cereus	17.0000±0.71^{a}	16.75±0.35^{b}	12.00±1.41^{ab}	9.65±1.20^{b}	7.00±0.00	0.00±0.00
L. plantarum strain ci-4w	*P. aeruginosa*	16.50±0.71^{b}	15.40±0.85^{b}	10.20±0.42^{a}	11.25±1.06^{b}	7.00±0.00	0.00±0.00
	*E. aerogene*s	18.25±0.35^{c}	16.75±0.35^{b}	14.75±1.06^{b}	10.50±0.71^{b}	0.00±0.00	0.00±0.00
	B. cereus	13.50±0.00^{a}	11.95±0.07^{a}	11.50±1.41^{ab}	7.50±0.71^{a}	0.00±0.00	0.00±0.00
L. sakei strain MLS1	*P. aeruginosa*	18.75±0.35^{b}	17.25±0.35^{b}	15.50±0.71^{b}	9.00±1.41^{a}	10.00±0.00^{b}	7.00±0.00^{b}
	E. aerogenes	14.50±0.00^{a}	12.85±1.20^{a}	14.75±1.06^{b}	12.90±0.14^{b}	7.50±0.71^{a}	0.00±0.00^{a}
	B. cereus	20.10±0.85^{b}	18.50±0.71^{b}	10.25±1.06^{a}	10.75±1.06^{ab}	10.75±0.35^{b}	7.25±0.35^{b}

Values are presented as means ± standard deviation and mean values in the same row having different superscript letters (a,b,c) are significantly different (p<0.05).

medium, temperature and incubation conditions or storage time. Wang et al. (2010) reported that cell aggregation and medium composition can affect bacteriocin production by LAB. This recent pH optimization studies indicate that bacteriocins produced by *L. amylolyticus* strain L6, *L. plantarum* strain ci-4w and *Lactobacillus sakei* strain MLS1 proved to have high stability at pH 5, 6 and 7; the bacteriocin activity was found to be highest at pH 7 with highest significant ($p < 0.05$) zone of inhibition of 19.40±071 mm, whereas considerable decrease was observed at both acidic (pH 4) as well as alkaline (pH 8 to 9) (Figures 8 to 10) which cohere with the report of Tambekar and Bhutada (2010) that bacteriocin were stable in acidic to neutral range, that is, from pH 3.0 to 7.0 but it became inactive in alkaline range at pH 9.0. Holzapfel et al. (2010) also reported that *L. plantarum* excreted other compounds such as bacteriocins that inhibited the growth of pathogens. However, this present study also indicates the strong biopreservation potential of these bacteriocins which may be used to extend the shelf life of food and also implies that bacteriocin obtained from *L. amylolyticus* strain L6, *L. plantarum* strain ci-4w and *L. sakei* strain MLS1 will be effective against both Gram positive and Gram negative bacteria such as *P. aeruginosa, E. aerogenes* and *Bacillus cereus* with maximum activity at neutral pH. This is similar to the report by Adebayo and Famurewa (2002) who opined that bacteriocin of *Lactobacillus* were active over a wide range of pH 2 to 6 and it is the optimum pH range for good inhibitory activity of bacteriocin from *Lactobacillus* strains against a wide range of various pathogenic organisms, while inactivation occurred mostly at pH 12.

Exposure of the crude bacteriocin to heating temperature had significant effects (P<0.05) on the antibacterial activity of the crude bacteriocin in this

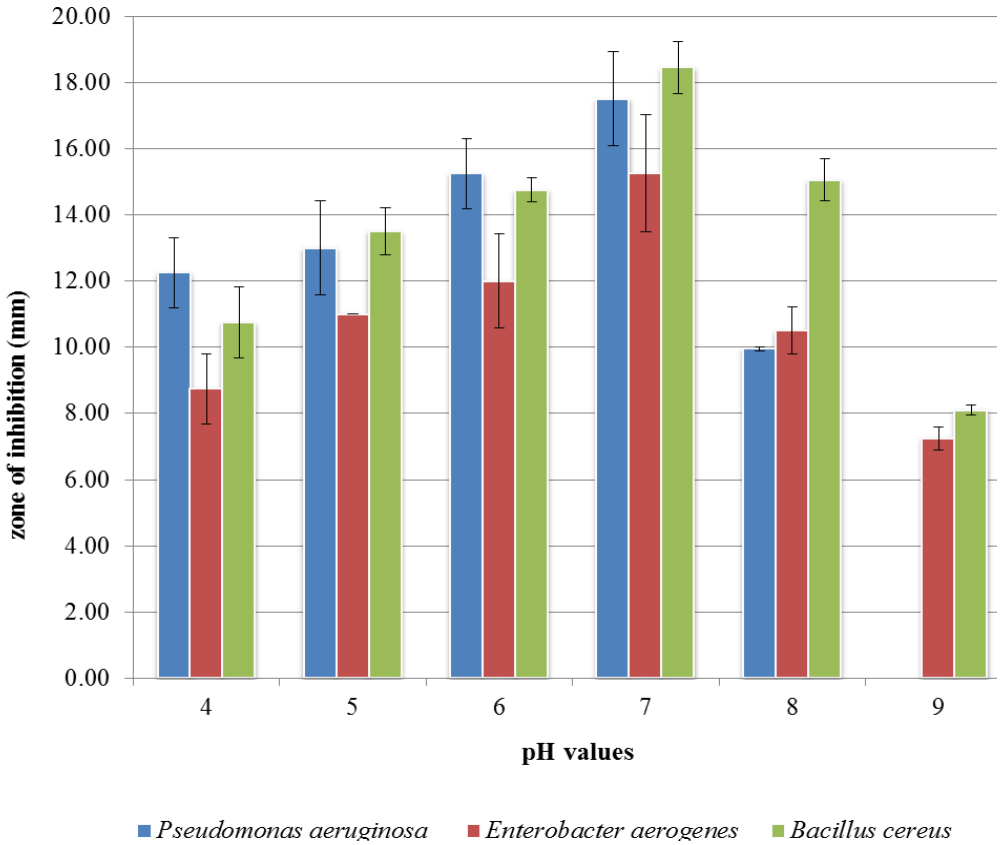

Figure 9. Effect of pH on crude bacteriocin activity of *Lactobacillus Plantarum* strain ci-4w.

present investigation. The result showed that the bacteriocin activity of *L. amylolyticus* strain L6 and *L. plantarum* strain ci-4w was most stable at 60°C for 10 min against all the indicator organisms although its resistance was observed up to 80°C for 10 min with partial loss of activity. This is in accordance with the work of Heredia-Castro et al. (2015) which showed that the antimicrobial activity of crude bacteriocin against *S. aureus*, *E. coli*, *Salmonella* Typhimurium, and *Listeria innocua* was stable at 65°C for 30 min but antimicrobial activity decreased to some extent at 100°C for 30 min and was most unstable at 121°C for 15 min. However, this present study exhibits that bacteriocin produced by *L. sakei* strain MLS1 showed thermo-stability over a wide range of temperature from 40 to 80°C for 10 min. This bacteriocin maintained full stability after heat treatment at 40, 60 and 80°C for 10 min but its efficacy decreased with the continuous increase in temperature, supporting the work of Padmavathi and Radha (2015). This result is in agreement with Veskovic-Moracanin et al. (2010) who stated that results of the examination of high temperatures on the activity of bacteriocin isolated from *L. sakei* I 154 implied its emphasized thermo resistance. Similar results by Ageni et al. (2017) and Ponce et al. (2008) reported that a number of bacteriocins produced by *Lactobacillus* strains were resistant at 100°C for 15 min.

The storage stability of crude bacteriocin by *L. amylolyticus* strain L6, *L. plantarum* strain ci-4w and *L. sakei* strain MLS1 was affected as different zones of inhibition were observed against the indicator organisms at varied storage time (h) (Table 10). Maximum activity was noted at 24 and 48 h storage period ranging from 9.75±1.06 to 18.50±0.71 mm against the food spoilage microorganisms. There were significant differences between the various storage times (at $P<0.05$). There were observable reduction bacteriocin activities as incubation time prolonged. This result was in total agreement with Onwuakor et al. (2014) who observed reduction of crude supernatant activities as incubation time dropped, they reported that optimum crude supernatant production was observed after 72 h judged by the zones of inhibition against the indicator. Whereas Tulini et al. (2011) observed a reduction of the bacteriocin production at 96 h when compared with the control incubation (24 h). This present result is in complete agreement with Obi (2015) and Elayaraja et al. (2014), who reported that the highest bacteriocin activity of *Lactobacillus tucceti* CECT 5920 was recorded within the first 1to 3 days against *S. aureus* NCTC 8325 and *E. coli*

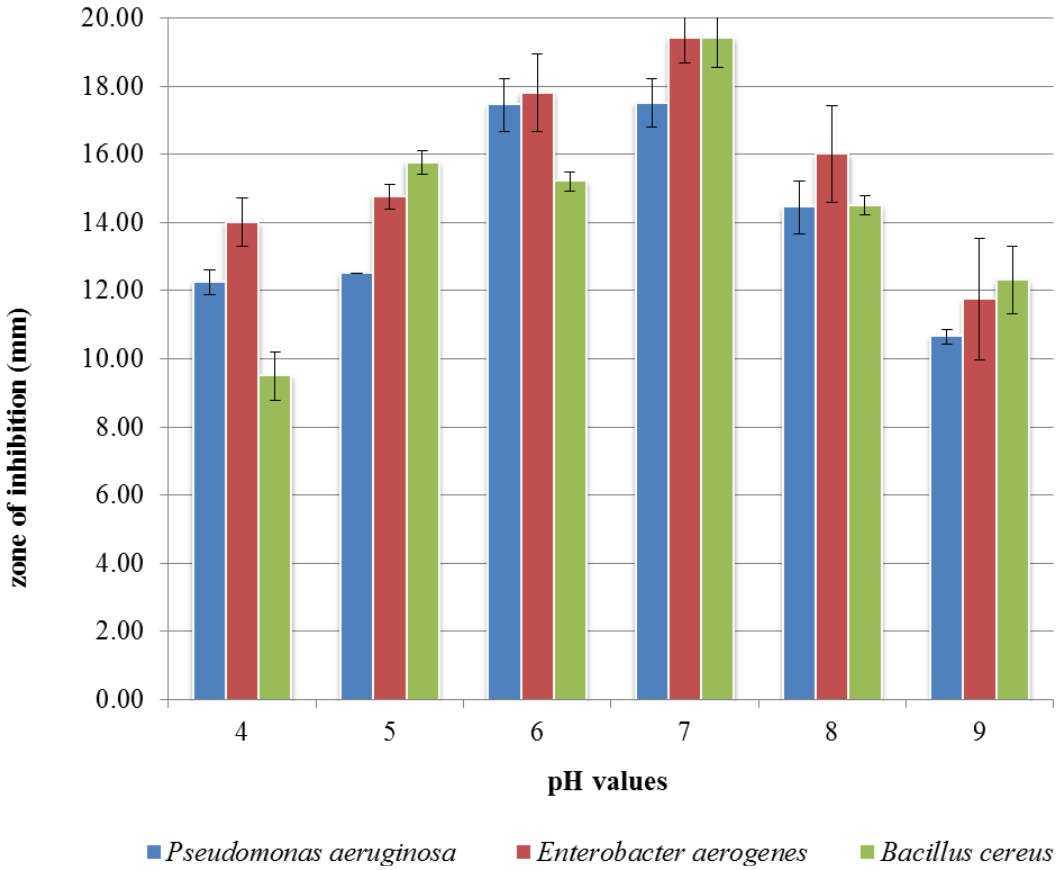

Figure 10. Effect of pH on crude bacteriocin activity of *Lactobacillus sakei* strain MLS1.

0157:H7.

Conclusion

In this study, the selected fermented food products (Ogi and Fufu) contain several strains of Lactobacilli, which are capable of producing antimicrobial compounds such as bacteriocins and have potential for exopolysaccharide (EPS) production. Among the isolated LAB from Ogi and Fufu, *L. amylolyticus* strain L6, *L. plantarum* strain ci-4w and *L. sakei* strain MLS1 exhibit good *in vitro* probiotic and biopreservative capacities. These strains were found to have wide pH tolerance, varying bile salt tolerance and antibacterial activities. The antibacterial activity shown by these strains could be useful to control undesirable contaminations in food industries. However, elimination of acid and hydrogen peroxide effect in cell free supernatant did not have any effect on the inhibitory activity of the bacteriocin rather the partially purified bacteriocin produced from *L. amylolyticus* strain L6, *L. plantarum* strain ci-4w and *L. sakei* strain MLS1 were found to be active against Gram positive (*B. cereus*) and Gram-negative bacteria (*P. aeruginosa* and *E aerogene*) suggesting its broad spectrum of activity and when harnessed under different culture conditions showed resistance to acidic pH more than basic pH, heat stable and retain its activity for a longer period which in turn will increase the shelf life of food which is considered an important criteria to be used in the modern food industry as biopreservative.

Therefore, *L. amylolyticus* strain L6, *L. plantarum* strain ci-4w and *L. sakei* strain MLS1 may be recommended as potential bacteriocin producers in the pharmaceutical industries to replace conventional antibiotics in combating pathogens that are vastly acquiring antimicrobial resistance and in the food processing industries to enhance extension of shelf life of food products; also, to replace the use of chemical preservatives as many chemicals used for the inactivation of food spoilage organisms and pathogens in order to preserve food products for longer period is being declined due to undesirable effects such as alteration of organoleptic and nutritional properties of food and their adverse toxic effects on human body system. Moreover, these strains can be used as starter culture or protective cultures to improve the hygiene, quality and increased safety of the food products by inhibiting the food borne pathogens and

spoilage microorganisms.

These results provided a basis for performing future studies on more purification steps of the bacteriocins for application as food preservative; also, assessment of *in vivo* probiotic characteristics of these potential *Lactobacillus* strains is encouraged.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

REFERENCES

Abegaz K (2007). Isolation, characterization and identification of lactic acid bacteria involved in traditional fermentation of borde, an Ethiopian cereal beverage. African Journal of Bacteriology 6(12):1469-1478.

Achi OK, Akomas NS (2006). Comparative Assessment of Fermentation Techniques in the Processing of Fufu, a Traditional Fermented Cassava Product. Pakistan Journal of Nutrition 5(3):224-229.

Adak K, Long S, O'Brien S (2002). Trends in indigenous foodborne disease and deaths. England and Wales: 1992 to 2000. Gut 51(6):832-841.

Adebayo CO, Famurewa O (2002). Antimicrobial effectiveness of in-house-use concentration of disinfectants in a Nigerian hospital. I Technolscience 6(1):17-20.

Adesokan IA, Odetoyinbo BB, Okanlawon BM (2009). Optimization of Lactic Acid Production by Lactic Acid Bacteria Isolated from Some Traditional Fermented Food in Nigeria. Pakistan Journal of Nutrition 8(5):611-615.

Ageni LV, Ajibade GA, Yerima B, Appah J (2017). Shelf life extension study of ogi and fufu using bacteriocin isolated from *Lactobacillus acidophilus* of fermented dairy products. African Journal of Microbiology Research 11(32):1286-1293

Amara S, Zadi-Karam H, Karam N (2019). Selection of *Lactobacillus* strains newly isolated from Algerian camel and mare fermented milk for their *in vitro* probiotic and lipolytic potentials. African Journal of Biotechnology 18(30):882-894.

Ananou S, Maqueda M, Martínez-Bueno M, Valdivia E (2007). Biopreservation, an ecological approach to improve the safety and shelf-life of foods In:.Communicating Current Research and Educational Topics and Trends in Applied Microbiology. A. Méndez-Vila (Ed.), Formatex.

Anumudu CK, Omeje FI, Obinwa GN (2018). Microbial succession pattern in Ogi fermentation. International Journal of Advanced Research in Biological Sciences 5(7):247-251

Ayoade F, Paulina OA, Kellanny SA, Yeitarere AA, Titilayo OA, Scott OF, Onikepe F (2018). The predominant lactic acid microorganisms and proximate composition of spontaneously fermented gari and fufu, cassava food products. Annual Research and Review in Biology 1-12.

Bali V, Panesar S, Bera B (2011). Isolation, Screening and Evaluation of Antimicrobial Activity of Potential Bacteriocin Producing Lactic Acid Bacteria Isolate. Microbiology Journal 1(3):113-119

Begley M, Gahan CGM, Hill C (2005). The interaction between bacteria and bile. FEMS Microbiology Reviews 29(4):625-651.

Bezkorvainy A (2001). Probiotics: Determinants of survival and growth in the gut. The American Journal of Clinical Nutrition 73(2):399s-405s.

Broberg A, Jacobsson K, Ström K, Schnürer J (2007). Metabolite profiles of lactic acid bacteria in grass silage. Applied and Environmental Microbiology 73(17):5547-5552.

Brown AC, Valiere A (2004). "Probiotics and Medical Nutrition Therapy". Nutrition in clinical care: an official publication of Tufts University 7(2):56-68.

Bukola CA, Abiodun AO (2008). Screening of Lactic Acid Bacteria Strains Isolated from Some Nigerian Fermented Foods for EPS Production. World Applied Sciences Journal 4(5):741-747.

Chan ES, Zhang Z (2005). Bioencapsulation by compression coating of probiotic bacteria for their protection in an acidic medium. Process Biochemistry 40:3346-3351.

Charlier CM, Cretenet S, Even Y, Le Loir Y (2009). Interactions between *Staphylococcus aureus* and lactic acid bacteria: An old story with new perspectives. International Journal of Food Microbiology 131(1):30-39.

Cleveland J, Montville TJ, Nes IF, Chikindas ML (2001). Bacteriocins: safe, natural antimicrobials for food preservation. International Journal of Food Microbiology 71(1):1-20.

De Martinis E, Bernadette D, Franco B (2001). Inhibition of *Listeria Monocytogenes*in a pork product by a *Lactobacillus sake* strain. International Journal of Food Microbiology 42(1-2):119-126.

Dinev T, Beev G, Tzanova M, Denev S, Dermendzhieva D, Stoyanova A (2017). Antimicrobial activity of *lactobacillus plantarum* against pathogenic and food spoilage microorganisms: A Review. Bulgarian Journal of Veterinary Medicine 21(3).

Dunne C, O'Mahony L, Murphy L, Thonton G, Morrissey D, O'Halloran S, Feeney M, Flynn S, Fitzgerald G, Daly C, Kiely B, O'Sullivan GC, Shanahan F, Collins JK (2001). In vitro selection criteria for probiotic bacteria of human origin: correlation with in vivo findings. The American Journal of Clinical Nutrition 73(2):386s-392s.

Elayaraja S, Annamalai N, Mayavu P, Balasubramanian T (2014). Production, purification and characterization of bacteriocin from *Lactobacillus murinus* AU06 and its broad antibacterial spectrum. Asian Pacific Journal of Tropical Biomedicine 4:S305-S311

Enan G, El-Essawy A, Uyttendaele M, Debevere J (1996). Antibacterial activity of *Lactobacillus plantarum* isolated from dry sausage: characterization production and bactericidal action of plantaricin UG1. International Journal of Food Microbiology 30:189-215.

Eze EI, Echezona BC, Uzodinma EC (2011). Isolation and identification of pathogenic bacteria associated with frozen mackerel fish (*Scomber scombrus*) in a humid tropical environment. African Journal of Agricultural Research 6(8):1947-1951

Garcha S (2018). Control of food spoilage molds using *lactobacillus* bacteriocins. Journal of Pure and Applied Microbiology 12(3):1365-1373.

Garcia EF, Luciano WA, Xavier DE, da Costa WC, de Sousa OK, Franco OL (2016). Identification of lactic acid bacteria in fruit pulp processing byproducts and potential probiotic properties of selected *Lactobacillus* strains. Frontiers in Microbiology 7:1371.

Garcia P, Rodriguez L, Rodriguez A, Martinez B (2010). Food biopreservation: Promising strategies using bacteriocins, bacteriophage and endolysins. Trends in Food Science and Technology 21(8):373-382..

Gong H, Meng, X, Wang H (2010). Plantaricin MG active against Gram-negative bacteria produced by *Lactobacillus plantarum* KLDS1.0391 isolated from "Jiaoke", a traditional fermented cream from China. Food Control 21(1):89-96.

Harrigan WF, McCance ME (1996). Laboratory methods in Microbiology. Academic press: London, p. 342.

Hassanzadazar H, Ehsani A, Mardani K, Hesari J (2012). Investigation of antibacterial, acid and bile tolerance properties of lactobacilli isolated from Koozeh cheese. Veterinary Research Forum 3(3):181-185.

Hata T, Tanaka R, Ohmomo S (2010). Isolation and characterization of plantaricin ASM1: A new bacteriocin produced by *Lactobacillus plantarum* A-1. International Journal of Food Microbiology 137(1):94-99.

Hati S, Mandal S, Prajapati JB (2013). Novel starters for value added fermented dairy products. Current Research in Nutrition and Food Science Journal 1(1):83-91.

Heredia-Castro PY, Méndez-Romero JI, Hernández-Mendoza A, Acedo-Félix E, González-Córdova AF, Vallejo-Cordoba B (2015). Antimicrobial activity and partial characterization of bacteriocin-like inhibitory substances produced by *Lactobacillus* spp. isolated from artisanal Mexican cheese. Journal of Dairy Science 98(12):8285-8293.

Hleba L, Císarová M, Glinushkin A, Laishevtcev A, Derkanosova A, Igor P, Shariati MA (2021). Physicochemical, functional and sensory properties of acha-tamba based ogi enriched with hydrolysed soy peptides. Journal of Microbiology, Biotechnology and Food Sciences

9(4):823-830.
Holt JG, Krieg NR, Sneath PH, Stalely JT, Williams ST (1994). Bergey's manual of determinative bacteriology. Bergey's Manual of Determinative Bacteriology. Williams and Wilkins.
Holzapfel WH, Cho G, Huch M, Franz CMP (2010). Genetic Analysis of the Plantaricin Efl. Locus of *Lactobacillus plantarum* PCS20 reveals an unusual plantaricin E gene sequence as a result of mutation. International Journal of Food Microbiology 141:5117-S124.
Ibeabuchi JC, Olawuni IA, Iheagwara MC, Ojukwu M, Ofoedu CE (2014). Microbiological Evaluation of 'Iru' and 'Ogiri-Isi' Used As Food Condiments. Journal of Environmental Science, Toxicology and Food Technology 8(8):45-50.
Inyang CU, Idoko CA (2006). Assessment of the quality of Ogi made from malted millet. African Journal of Biotechnology 5(22):2334-2337
Isolauri E, Salminen S, Ouwehand A (2004). Microbial-gut interactions in health and disease, Probiotics. Best Practice and Research Clinical Gastroenterology 18(2):299-313.
Jena PK, Trivedi D, Thakore K, Chaudhary H, Giri SS, Seshadri S (2013). Isolation and characterization of probiotic properties of lactobacilli isolated from rat fecal microbiota. Microbiology and Immunology 57(6):407-416.
Karthikeyan V, Santosh SW (2009). Isolation and partial characterization of bacteriocin produced from *Lactobacillus plantarum*. African Journal of Microbiology Research 3(5):233-239.
Kumar S, Stecher G, Li M, Knyaz C, Tamura K (2018). MEGA X: Molecular Evolutionary Genetics Analysis across computing platforms. Molecular Biology and Evolution 35(6):1547-1549.
Leyer GL, Johnson EA (1993). Acid adaptation induces cross-protection against environmental stresses in *Salmonella typhimurium*. Applied and Environmental Microbiology 59(6):1842-1847.
Mandal S, Puniya AK, Singh K (2006). Effect of alginate concentration on survival of encapsulated *Lactobacillus casei* NCDC-298. International Dairy Journal 16(10):1190-1195.
Martinez R, Wachsman M, Torres N, LeBlanc J, Todorov S, de Melo Franco B (2013). Biochemical, antimicrobial and molecular characterization of a noncytotoxic bacteriocin produced by *Lactobacillus plantarum* ST71KS. Food Microbiology 34(2):376-381.
Martoni C, Bhathena J, Jones ML, Urbanska AM, Chen HM, Prakash S (2007). Investigation of microencapsulated BSH active *Lactobacillus* in the simulated human GI tract. Journal of Biomedicine and Biotechnology 12:1-9.
Maurya P, Thakur L (2012). Inhibition spectrum, purification and characterization of bacteriocin from *Leuconostoc NT*-1. Current Science 103(12):1405-1407.
Merzoug M, Dalache F, Zadi Karam H, Karam NE (2016). Isolation and preliminary characterisation of bacteriocin, produced by *Enterococcus faecium* GHB21 isolated from Algerian paste of dates "ghars". Annals of Microbiology 66(2):795-805.
Merzoug M, Mosbahi K, Walker D, Karam NE (2018). Screening of the Enterocin-Encoding Genes and Their Genetic Determinism in the Bacteriocinogenic *Enterococcus faecium* GHB21. Probiotics and Antimicrobial Proteins 11(1):325-331.
Mostefaoui A, Hakem A, Yabrir B, Boutaiba S, Badis A (2014). Screening for exopolysaccharide producing strains of thermophilic lactic acid bacteria isolated from Algerian raw camel milk. African Journal of Microbiology Research 8(22): 2208-2214
Nath S, Chowdhury S, Sarkar S, Dora C (2013). Lactic Acid Bacteria – A Potential Biopreservative In Sea Food Industry. International Journal of Advanced Research 1(6):471-475
Ngene AC, Onwuakor CE, Aguiyi JC, Ifeanyi VO, Ohaegbu CG, Okwuchukwu CP, Egbere JO (2019). Screening of Some Lactic Acid Bacteria Isolated from Selected Nigerian Fermented Foods for Vitamin Production. Advances in Microbiology 9(11):943-955.
Nwachukwu E, Ijeoma O (2010b). Isolation and characterization of Lactic Acid Bacteria associated with the fermentation of a cereal-based product for the development of a starter culture. Food 4(1):45-48
Nwokoro O, Chukwu BC (2012). Studies on Akamu, a traditional fermented maize food. Revista Chilena de Nutrición 39(4):180-184.
Obi CN (2018). In vitro screening for human probiotic potentials of *Lactobacillus tucceti* CECT 5920 and *Lactobacillus mindensis* TMW isolated from Nigerian Fermented foods. The Bioscientist Journal 6(1):13-30.
Odunfa SA, Adeyele S, (2000). "Preliminary Study of the Effect of Lactic Fermentation on the Rheology and pH of Ogi Porridge", Journal of Food Microbiology 20(25):71-75.
Ogunshe AA, Omotosho MA, Adeyeye JA (2007). In vitro Antimicrobial Characteristic of Bacteriocin Producing Lactobacillus strains from Nigeria Indigenous Fermented Food. African Journal of Biotechnology 6(4):445-453.
Onwuakor CE, Nwaugo VO, Nnadi CJ, Emetole JM (2014). Effect of Varied Culture Conditions on Crude Supernatant (Bacteriocin) Production from Four Lactobacillus Species Isolated from Locally Fermented Maize (Ogi). American Journal of Microbiological Research 2(5):125-130
Oyedeji O, Ogunbanwo ST, Onilude AA (2013). Predominant Lactic Acid Bacteria Involved in the Traditional Fermentation of Fufu and Ogi, Two Nigerian Fermented Food Products. Food and Nutrition Sciences 4(11):40-46.
Padmavathi T, Radha KR (2015). Purification, characterization and application of bacteriocin for improving the shelf-life of sprouts: An approach to Bio preservation. Indicator 8(9):10.
Patel A, Lindström C, Patel A, Prajapati JB, Holst O (2012). Probiotic properties of exopolysaccharide producing lactic acid bacteria isolated from vegetables and traditional Indian fermented foods. International Journal of Fermented Foods 1(1):87-101.
Ponce AG, Moreira MR, Del Valle CE, Roura SI (2008). Preliminary characterization of bacteriocin-like substances from lactic acid bacteria isolated from organic leafy vegetables. LWT-Food Science and Technology 41(3):432-441.
Radha KR, Padmavathi T (2015). Purification, characterization and application of bacteriocin for improving the shelf-life of sprouts: An approach to Bio preservation. International Journal of ChemTech Research 8(8):265-277
Rijkers GT, de Vos WM, Brummer RJ, Morelli L, Corthier G, Marteau P (2011). Health benefits and health claims of probiotics: Bridging science and marketing. British Journal of Nutrition 106 (9):1291-1296.
Ross P, Morgan S, Hill C (2002). Preservation and fermentation: past, present and Future. International Journal of Food Microbiology 79(1-2):3-16.
Sahadeva RPK, Leong SF, Chua KH, Tan CH, Chan HY, Tong EV, Wong SYW, Chan HK (2011). Survival of commercial probiotic strains to pH and bile. International Food Research Journal 18(4):1515-1522.
Saitou N, Nei M (1987). The neighbor-joining method: A new method for reconstructing phylogenetic trees. Molecular Biology and Evolution 4(4):406-425.
Salem AM (2012). Bio-Preservation Challenge for Shelf-Life and Safety Improvement of Minced Beef. Global Journal of Biotechnology and Biochemistry 7(2):50-60
Sharma N, Kapoor G, Neopaney B (2006).Characterization of a new bacteriocin produced from a novel isolated strain of *Bacillus lentus* NG121. Antonie Van Leeuwenhoek 89(3):337-343.
Sultana K, Godward G, Reynolds N, Arumugaswamy R, Peiris P, Kailasapathy K (2000). Encapsulation of probiotic bacteria with alginate-starch and evaluation of survival in simulated gastrointestinal conditions and in yoghurt. International Journal of Food Microbiology 62(1-2)):47-55.
Tambekar DH, Bhutada SA (2010). Acid and bile tolerance, antibacterial activity, antibiotic resistance and bacteriocins activity of probiotic *lactobacillus* species. Recent Research in Science and Technology 2(4):94-98
Todorov S, Dicks L (2005). *Lactobacillus plantarum* isolated from molasses produced bacteriocins active against Gram negative bacteria. Enzyme and Microbial Technology 36(2-3):318-326.
Todorov SD, Oliviera PS, Vaz-Velho (2012). Media Optimization of Bacteriocin ST22Ch Production by *Lactobacillus sakei* ST22Ch Isolated from Salpicao, a Traditional Meat-a product from Portugal, Chemical Engineering Transactions 27:283-288.
Tulini FL, Gomes BC, de Martinis EC (2011). Partial purification and characterization of a bacteriocin produced by *Enterococcus faecium* 130 isolated from mozzarella cheese. Food Science and Technology 31(1):155-159.
Van den Berg DJC, Smith A, Pot B, Ledeboer AM, Kerstens K, Verbakel

JMA, Verrips CT (1993). Isolation, screening and identification of lactic acid bacteria from traditional food fermentation processes and culture collections. Food Biotechnology 7(3):189-205.
Van Geel-Schuttená GH, Flesch F, ten Brink B, Smith MR, Dijkhuizen L (1998). Screening and characterization of Lactobacillus strains producing large amounts of exopolysaccharides. Applied Microbiology and Biotechnology 50(6):697-703.
Veskovic-Moracanin S, Obradovic, D, Velebit, Branka B, Marija S, Turubatovic L (2010). Antimicrobial properties of indigenous *Lactobacillus sakei* strain. Acta Veterinaria 60(1):59-66.
Vignolo G, Castellano P, Belfiore C, Fadda S (2008). A review of bacteriocinogenic lactic acid bacteria used as bio-protective cultures in fresh meat produced in Argentina. Meat Science 79(3):483-499
Wakil SM, Ajayi OO (2013). Production of lactic acid from Starchy-based food substrates. Journal of Applied Biosciences 71:5673-5681
Wang Q, Cui Y, Lackeyram D, Yuan L, Xu J, Wang W, Li Xu L (2010). Effect of cultural components on antimicrobial activity of bacteriocin produced by bacteria isolated from gut of poultry. African Journal of Microbiology Research 4(19):1970-1980.
Wilson BA, Thomas SM, Ho M (2011). The human vaginal microbiome. In: Nelson KE, editor. Metagenomics of the human body. Germany: Springer. pp. 91-115.

Vol. 21(5), pp.237-251, May 2022
DOI: 10.5897/AJB2022.17467
Article Number: 9654AE369208
ISSN: 1684-5315

http://www.academicjournals.org/AJB

ACADEMIC JOURNALS
expand your knowledge

African Journal of Biotechnology

Full Length Research Paper

New technique for improving fish packaging hygiene and prolonged shelf life

Nagwa T. Elsharawy[1,2*], Wafa A. Baabdullah[1] and Ali Alkaladi[1]

[1]Department of Biology, College of Science, University of Jeddah, Jeddah 21959, Saudi Arabia.
[2]Department of Food Hygiene, Faculty of Veterinary Medicine, New Valley University 72713, Egypt.

Received 11 February, 2022; Accepted 4 May, 2022

Zinc oxide nanoparticles (ZnO NPs) have received great attention due to their optical, physical, and antimicrobial properties. They have toxic effect against microbes without any effect on mammalians cells. They are used in several applications including extending the shelf life of food. The study aims to determine the minimum inhibitory concentrations of ZnO NPs against different aquaculture fish fungus species and their storage period. A total of 160 samples were collected from different types of aquaculture fish samples as follows: rabbitfish, bream, red mullet, saddle grouper, spangled emperor, gilthead seabream, mackerel fish, and Asian seabass. ZnO NPs activity against the isolated fungus species was evaluated by estimating minimum fungicidal inhibitory concentration and inhibition of fungal enzymes (amylase, protease, and lipase). The storage period of the fish in a package containing ZnO NPs was determined by estimating the sensory characteristics of the treated fish. The results obtained recorded the following fungus species from aquaculture fish samples: *Aspergillus niger* (gi: JX112703), *Aspergillus oryzae*, *Aspergillus awamori*, *Penicillium* species, *Aspergillus tubingensis*, *Trichosporon montevideense*, *A. niger* (gi: MG889596), and *Byssochlamys spectabilis*, respectively. This study is the first to apply ZnO NPs for fish preservation which have a powerful antifungal effect against all the isolated fungi. The study recommends using 3% ZnO NPs in fish packaging film; it inhibited most of the fungus species, extending the shelf life of most of the fish species to more than 15 days.

Key words: Zinc oxide nanoparticles, shelf life, fish preservation, minimum inhibitory concentration (MIC), antifungal, aquaculture fish fungus.

INTRODUCTION

Fish is an essential source of many necessary elements to human health such as protein, vitamins, and different nutrients (Khan et al., 2018). However, the average consumption of fish in Saudi Arabia is low, equivalent to 9 kg per person per year; while in Japan, a person consumes 60 kg per year. Saudi citizens have increased awareness about the importance of seafood and its reflection on human health. Saudi Arabia's aquaculture projects produce nearly 70,000 tons of fish, and the government is seeking to raise production to 600,000

*Corresponding author. E-mail: dr.nagwa2004@yahoo.com.

tons by 2030 (Rahman et al., 2017). Most researches indicated that fish reared in a polluted aquatic environment have a high susceptibility to different illnesses and contamination than those reared in non-polluted marine environments (Ngo et al., 2021). Depletion of aquatic oxygen, pH changes and the unusual increase of the aquatic organic content increase the microbial loads of fish (Cannas et al., 2020). Aquaculturing on polluted aquatic environment by chemical and/or biological contaminations affects the quality of harvested fish; it causes decreased metabolism, liver damage, ulceration, neoplasia, immunosuppression, hyperplasia in fish. It damages the epithelia, tail, fins and gills of fish. This decreases aquaculture production and/or results in production of low quality fish due to its high biological load. It also affects adversely the shelf life of fish (Javed et al., 2016; Ayalew and Fufa, 2018). The improper management, treatment and storage of fish lead to waste of about 50% of fish harvested annually (Chavan et al., 2011). The high distance between landing sites, markets, and consumption areas may cause a high economic loss. To reduce the wastage that occurs by oversupply and to prolong the storage life of fish, an effective novel technique for fish storage is required. The main cause of harvesting fish spoilage is fungal contamination, which appeared as discoloration, off-flavor, rotting, and textural quality of fish. This leads to loss of nutrient quality which causes huge economic loss and hazard to consumers' health (Magwaza et al., 2017; Rico-Munoz et al., 2018). The most encountered fungal genuses are *Aspergillus*, *Trichosporon*, *Penicillium*, and *Aspergillus* species especially *Aspergillus niger*, *Aspergillus oryzae*, and *Aspergillus tubingensis*. Meanwhile, the presence of pathogenic fungi may cause immunocompromised handlers and consumers to have aspergillosis. This has encouraged scientists to develop a technique to control food borne microorganisms (CODEX, 2009; Derrick, 2009). The contamination of fish by fungi decreases its shelf life which leads to waste of about 60% of the fish aquaculturing cost (Tacon, 2020). It also has environmental and financial cost impact due to the difficulty of disposing the spoiled fish (White, 2013).

Zinc oxide is used recently in food packaging and processing due to its safety, thermal stability and mechanical resistance. It improves the physical character of packaging materials (Duncan, 2011; Rajeshkumar, 2019). The antimicrobial properties of this metal are exaggerated when used as nanoparticles especially in food technology (Qasim, 2011). Zinc oxide nanoparticles (ZnO-NPs) are one of the most effective food packaging substances due to their binding patterns and interactions properties, moisture absorption, monolayer moisture, and solubility (Crona et al., 2020). They also have excellent physical properties such as permeability, desorption, degree elongation, tensile strength, and mechanical properties as food packaging substances (Swain et al., 2014; Paul et al., 2019). Few research works have been done on ZnO nanoparticles that spread through food. Although, the toxicological side effect of ZnO NPs needs more studies to determine their effects on food safety (Paul et al., 2019). Most scientists have studied the toxicity of ZnO NPCs. They found that they are non-toxic materials and have vital mineral supplement for the human body. They have selective toxicity against wide range of microbes that encourage their use as food additives (Stoimenov et al., 2002; Zhang et al., 2007). The US Food and Drug Administration (21CFR182.8991) reported ZnO as one of five safe Zn compounds (Bradley et al., 2011).

There was a surveillance shortage in fish spoilage fungus species in aquaculture fish generally and marketed fish species in Saudi Arabia. This encourages us to perform this study, which aimed to find additional knowledge to enhance proper control of the storage life of fish and fish product by determining the antifungal effect of different concentrations of ZnO NPs on different fish fungal contamination, the minimum inhibitory concentrations (MICs) of nanoparticles against fungal contamination of aquaculture fish sold in Jeddah, Saudi Arabia markets, and the storage period of fish using packages containing ZnO NPs.

MATERIALS AND METHODS

Sample collection

A total of 160 samples were collected from 8 types of aquaculture fish (20 fish from each type): rabbitfish "*Siganus rivulatus*", bream "*Pagrus pagrus*", red mullet "*Mullus surmuletus*", saddle grouper "*Najil P. pessuliferus*", spangled emperor "*Lethrinus nebulosus*", gilthead seabream "*Sparus aurata*", mackerel fish "*Scomberomorous commerson*", and Asian seabass "*Lates calcarifer*". The samples were freshly purchased and stored in ice box and refrigerated. All fish samples were collected from January to July 2021 and transferred to the laboratories of Collage of Science, University of Jeddah.

Fungal Isolations and Identification

The fish surface was disinfected by 1% formaldehyde; the fish was dipped into it from 1 to 5 min. Then, it was transferred to 70% alcohol, and washed with sterilized distilled water. About 1 g of the inner fish tissues were directly spread onto the Petri plates of potato dextrose agar (PDA) medium. Penicillin and streptomycin (50 mg/L) were added to the medium to avoid bacterial contamination. The medium was aseptically dispensed into sterile Petri dishes (Koh et al., 2000; Cao et al., 2015). Identification was done by observing the colony color and texture. It was stained with 0.05% trypan blue in lactophenol. Then, molecular identification was done by PCR detection (Javadi et al., 2012; Rico-Munzo et al., 2018).

Nanoparticle substances

Zinc Oxide NPs (70 ± 15 nm) were bought from Nano Gate (Creating New Scientific Horizons, Egypt). Original suspension of ZnO NP (12 mmol L^{-1}) was then diluted using PDA to make different concentrations of ZnO NPs: 1, 2, 3, and 5% and NP-free

solution [control] (He et al., 2011).

Antifungal test

Autoclaved PDA media with ZnO NPs at different concentrations "1, 2, 3, and 5%" were incubated at 25°C. Then, the antifungal activity was evaluated at the time intervals of 2, 4, 6, 9, and 12 days. The diameter of the fungus colonies was detected in triplicate plates as described previously (Fraternale et al., 2003).

Microbial culture

This was obtained by isolating pure fungus containing identified *Aspergillus* spp., *Penicillium* spp. and *Byssochlamys spectabilis.* It was tested for amylase production by starch plate method (Ross, 1976), protease and lipase by skim milk agar (Zaitz et al., 2004).

Amylase assay

About 25 g of starch agar medium was suspended in 1000 ml distilled water. About 4 mm of fungal culture was cut on a labeled plate and then incubated at 25°C/48 h with a drop of iodine solution for 30 s. The color of the medium changed because amylase is a starch hydrolyzing enzyme (Ross, 1976).

Protease assay

About 51.5 g of skim milk agar plate was suspended in 1000 ml of distilled water. The fungi were inoculated separately. Hydrolysis results were obtained in the clear zones (zone of hydrolysis) around the fungal colonies at the end of the incubation at 25°C for 48 h (Ali, 1992).

Lipase assay

Agar (2.5%) was added to 2% Tween 20-80 and 0.01% Victoria Blue B (or other indicators). About 1 cm of circular well around the different isolated fungi was grown at 30°C. Lipolytic microorganisms were picked out from the culture plates (Samad et al., 1989).

Determination of the storage period of fish using packages containing NPs

Red Sea fish (about 300 g) was purchased and transferred into a sterile glass container under sanitized conditions. It was packaged with polyethylene (PE) films previously prepared with different concentrations of ZnO (1, 2, 3, and 5%), which were sprayed on the PE surface ("10×150 cm"). It was left to dry at room temperature (26°C). Each 100 g of fish was wrapped by each package film concentration, stored under dark and cool conditions (4°C) and compared to the fish wrapped by uncoated PE film package. The shelf life of each fish type was observed and the fungal growth was tested by using PDA. The fish were incubated between 28±2°C/examined daily for 7, 14, and 18 days and 18±2°C/examined daily for 18 days. The experiments were repeated at least 5 times using each concentration for results confirmation (Ebrahimiasl and Rajabpour, 2015; Al-Naamani et al., 2016).

Sensory evaluation of treated fish

Four samples of different fish treatments were covered in small dishes. The samples were evaluated for the following parameters: acceptability by odor intensity, appearance, flavor and after cooking taste, juiciness, tenderness, off-flavor, and off-odor. An eight-point of scoring scale (8 = extremely intense/tender/juicy, 7 = very intense/tender/juicy, 6 = moderately intense/tender/juicy, 5 = slightly intense/ tender/juicy, 4 = slightly bland/tough/dry, 3 = moderately bland/tough/dry, 2 = very bland/tough/dry, 1 = extremely bland/tough/dry) was applied for odor and flavor intensity, tenderness, and juiciness, respectively (Sallam, 2007).

Statistical analysis

The statistical program, SPSS version 16 for window was used for the determination of means, standard error, and analysis of variance (ANOVA) using the one way (mean at the significance level of ($P<0.05$). Statistical significance was tested at the 5% level of significance in this study (SPSS 16, 2007).

RESULTS

Prevalence of different fungal genera in the examined fish samples

The total positive result was 147/160 (92%) from the total examined samples, while the negative result was about 13/160 (8%). The positive prevalence results were found in (Table 1) *Aspergillus niger* (gi:JX112703) followed by *A. oryzae, Aspergillus awamori* and *Penicillium* spp*., A. tubingensis, Trichosporon montevideense,* and *A. niger* (gi:GM889596). The fungus with the lowest incidence was *B. spectabilis*. The different types of fungi found in the different fish species are arranged as follows: Asian seabass "*L. calcarifer*" and *A. oryzae*. In the case of Red Sea bream "*P. pagrus*", the fungus species with the highest incidence was *A. niger* (gi:JX112703). Rabbitfish "*S. rivulatus*" had *A. niger* (gi:JX112703). Spangled emperor "*L. nebulosus*" reported the highest incidence of *Penicillium* spp. Gilthead seabream "*S. aurata*" samples reported *A. oryzae*. Mackerel fish "*Scomberomorous commerson*" samples had *A. niger* (gi:JX112703). Red mullet "*M. surmuletus*" samples reported *T. montevideense*. Saddle grouper "*Najil P. pessuliferus*" samples reported *A. niger* (gi:JX112703).

Identification of isolated fungal genera

Figure 1 describes the isolated fungal species with their phylogenetic molecular tree and most related genera. *Aspergillus niger* (gi:JX112703) colonies were spherical, had thick walls, densely black to dark brown conidia, and white mycelia. Microscopically, *A. niger* (gi:JX112703) spores appeared as dark brown/carbon black. It was grouped into 5 groups with 100% genetic similarity*. A. niger* (gi:MG889596) colonies had compact white base; they have condensed black conidial heads which enlarge and roughen with maturity. *A. niger* (gi:MG889596) is a filamentous fungus that looks like a plant structure. It was

Table 1. Prevalence of different fungal Spoilage species isolated from fish samples.

Fish type	Fungus Types							
	Aspergillus species					*Penicillium* spp.	*Byssochlamys spectabilis*	*Trichosporon montevideense*
	A. niger (gi:JX112703)	*A. niger (gi:MG889596)*	*A. awamori*	*A. oryzae*	*A. tubingensis*			
Asian seabass "*Lates calcarifer*"	4 (20)	2 (10)	4 (20)	6 (30)	4 (20)	2 (10)	3 (15)	2 (10)
Bream "*Pagrus pagrus*"	10 (50)	3 (15)	5 (25)	1 (05)	0 (00)	2 (10)	0 (00)	5 (25)
Rabbitfish "*Siganus rivulatus*"	13 (65)	0 (00)	4 (20)	2 (10)	4 (20)	0 (00)	2 (10)	0 (00)
Spangled emperor "*Lethrinus nebulosus*"	5 (25)	4 (20)	3 (15)	2 (10)	4 (20)	6 (30)	0 (00)	0 (00)
Gilthead seabream "*Sparus aurata*"	0 (00)	0 (00)	0 (00)	13 (65)	2 (10)	6 (30)	0 (00)	0 (00)
Mackerel fish "*Scomberomorous commerson*"	4 (20)	0 (00)	1 (05)	0 (00)	1 (05)	2 (10)	0 (00)	0 (00)
Red mullet "*Mullussurmuletus*"	2 (10)	1 (05)	1 (05)	0 (00)	0 (00)	0 (00)	0 (00)	5 (25)
Saddle Grouper "*Najil P. pessuliferus*"	3 (15)	1 (05)	0 (00)	3 (15)	0 (00)	0 (00)	0 (00)	0 (00)
Total	41/147 (28.0)	11/147 (8.0)	18/147 (12.3)	27/147 (18.4)	15/147 (9.4)	18/147 (12.3)	5/147 (3.4)	12/147 (8.2)

grouped into 5 groups with 100% genetic similarity.

A. awamori colonies appeared as visible peripheral growth, and had smooth surface (light-yellow); they had several conidia which are black, whitish-yellow, and dark brown with wavy slight surface. Microscopically, *A. awamori* appeared as single and aggregated colonies that resemble plant-like filamentous fungi. It was grouped into 7 groups with 99% genetic similarity. *A. oryzae* colony surface appeared as olive-green or green; it has white conidia and white mycelia. Microscopically, *A. oryzae* colonies appeared as pale grey to black; they have conidial heads with a coarse wall and short column. *A. oryzae* was grouped into 6 groups with 100% genetic similarity.

Macroscopically, *A. tubingensis* colonies appeared as yellow at the beginning and became flat, granular, and bright to dark yellow-green with radial grooves. They are finely wrinkled, globular, and have warty conidia. Microscopically, *A. tubingensis* appeared as single aggregated pale yellowish-green colonies; they have radiated conidia heads with coarse roughened wall and loose columns. *A. tubingensis* was grouped into 4 groups with 99% genetic similarity. Macroscopically, *Penicillium* spp. colonies have woolly texture. Initially, their color is white then turns to yellowish or pinkish; they have olive-gray or white conidia. Microscopically, *Penicillium* spp. has branched hyaline or simple conidia with cup-shaped phialides; they have brush-like clusters at the tips, which are known as "penicilli". *Penicillium* spp. were grouped into 4 groups with 99% genetic similarity. Macroscopically, *B. spectabilis* appeared as wheat-colored conidia with wrinkled yellowish to light brown; it has wooly to downy texture, and brown to pale surface. Microscopically, the individual aggregate of *B. spectabilis* has branched hyaline with brush like tip; it is ovoid with elongated, solitary chains. *B. spectabilis* was grouped into 8 groups with 99% genetic similarity. *T. montevideense* macroscopically appeared as dense pure white mycelia and conidia. From the PDA plate it appeared as light-yellow wrinkled reverse with globose vesicle and radiated conidia head. Microscopically, *T. montevideense* appeared as yeast-like colonies with septate hyphae, arthroconidia, and budding cells. *T. montevideense* was grouped into 4 groups with 99% genetic similarity.

Effect of ZnO nanoparticles on the different types of isolated fungi (*in vitro*)

The effect of different concentrations of ZnO NPs (1, 2, 3, and 5%) on the isolated fungi (*in vitro*) was compared to that of antifungal drug on the fungal species (Table 2 and Figure 2). The results revealed that 5% concentration of ZnO NPs was more effective than the antifungal drug followed by 3, 2, and 1%, respectively. 5% ZnO NPs inhibited 2.90 cm *A. niger* (gi:JX112703), which was the highest. This is followed by 3% ZnO NPs which inhibited 2.50 cm *A. niger* (gi:JX112703). The antifungal drug inhibited about 2.30 cm *A. niger* (gi:JX112703). 2% ZnO NPs inhibited 2.20 cm *A. niger* (gi:JX112703). 1% ZnO NPs inhibited

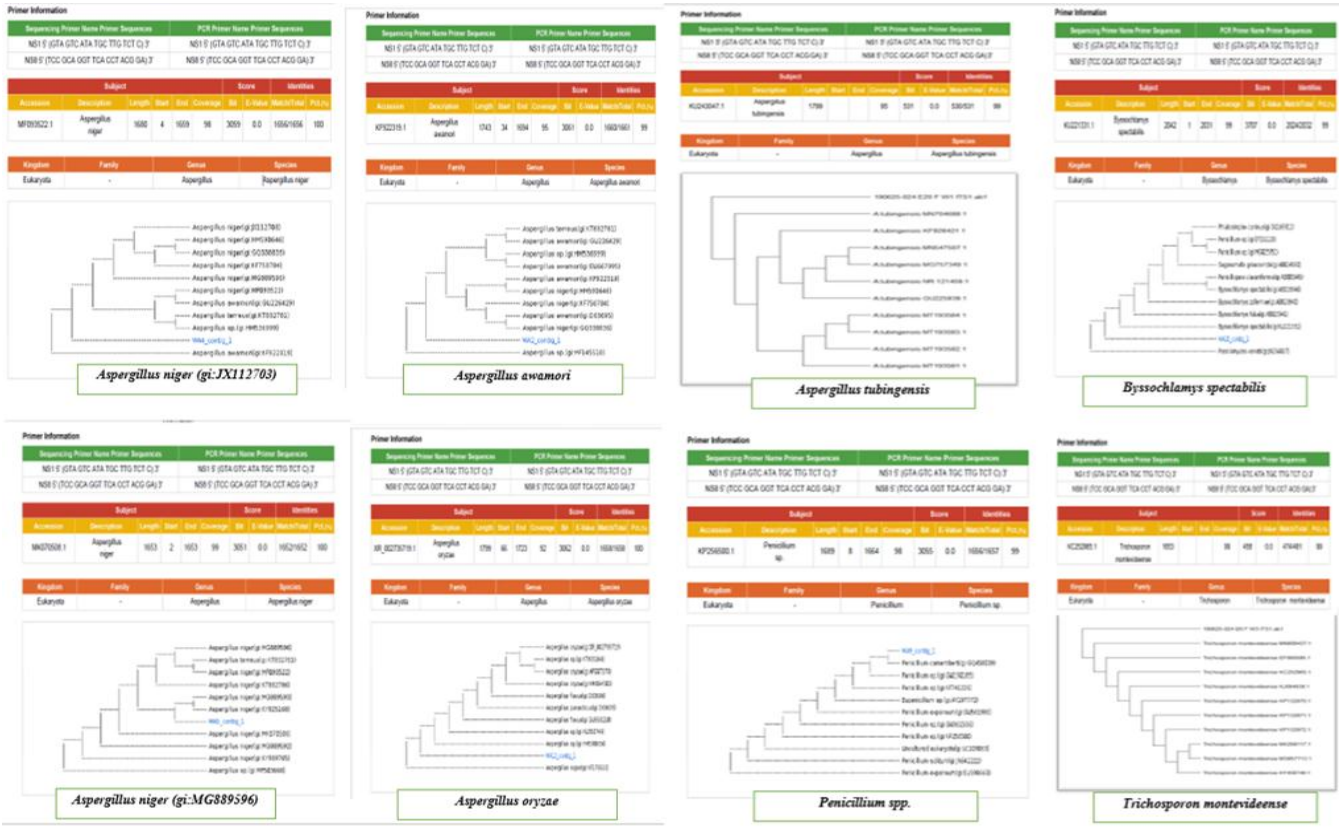

Figure 1. Phylogenetic molecular tree of the selected isolate and the most related gene isolate and the most related gene.

about 1.90 cm *A. niger* (gi:JX112703), which was the lowest inhibition effect. Nearly similar effect was detected against *A. niger* (gi:MG889596); the inhibition effect began gradually with 5% ZnO NPs inhibiting about 2.80 cm *A. niger* (gi:MG889596), 3% ZnO NPs inhibited 2.20 cm *A. niger* (gi:MG889596). 2% ZnO NPs and the antifungal drug had similar inhibition against the fungus (about 2.00 cm). The lower inhibition zone was measured with 1% ZnO NPs, which inhibited 1.70 cm *A. niger* (gi:MG889596).

About 2.30 cm *A. awamori* was inhibited by 5% ZnO NPs, which was the highest inhibition effect followed by 3% ZnO NPs and the antifungal drug which inhibited 2.00 cm of the fungus. 2% ZnO NPs inhibited about 1.90 cm of the fungus. The lowest inhibition zone was measured with 1% concentration of ZnO NPs, which inhibited 1.7 cm of the fungus. *A. oryzae* was one of the most resistant fungi although1.90 cm was inhibited by 5% ZnO NPs; while the inhibition zone was 1.80 cm in the case of 3% ZnO NPs and the antifungal drug. Lower inhibition zone measured by 2 and 1% was as follows: 1.50 and 1.30 cm, respectively. *A. tubingensis* was one of the lower resistant fungi. The inhibition effect was about 1.90, 1.70, 1.60, 1.40 and 1.30 cm in case of 5, 3, 2 and 1% ZnO NPs, and antifungal drug, respectively.

Penicillium spp. has the highest inhibition effect: 5 and 3% ZnO NPs inhibited it by 2.80 and 2.50 cm. The inhibition zone was equal in the case of the antifungal and 2% ZnO NPs concentration, which inhibited 1.90 cm of the fungus. The lowest inhibition effect was recorded in 1% ZnO NPs which inhibited 1.80 cm of the fungus. 5, 3, and 2% ZnO NPs had the highest inhibition effect against *B. spectabilis* (3.20, 2.50, and 2.20 respectively). Antifungal drugs recorded the same inhibition zone (1.50 cm) with 1% ZnO NPs. 5, 3, and 2% ZnO NPs inhibited *T. montevideense* by 2.80, 2.50, and 2.00 cm; while 1.90 and 1.50 cm was inhibited by the antifungal drug and 1% ZnO NPs, respectively.

Effect of ZnO NPs on isolated fungal enzymes

ZnO NPs affect the growth of fungi by attacking their cell structure and/or fungal enzymes. Table 3 shows the effect of adding ZnO NPs on amylase enzyme secreted by different fungal species in the case of *Aspergillus* spp. The fungal growth was about 14 mm in the control samples, while the growth decreased to 4 mm after the antifungal drug (1, 2, 3 and 5%) was added. The amylase activity decreased by about 19 mm in *Aspergillus* spp. plates, while it decreased to 8 mm only in the antifungal drug. 7 mm amylase was inhibited after adding 1 and 2%

Table 2. Effect of ZnO nanoparticles on different types of isolated fungi (*in vitro*).

Fungus	1%				2%				3%				5%				A. F			
	Minimum (cm)	Maximum (cm)	Mean (cm)	S.E. ±	Minimum (cm)	Maximum (cm)	Mean (cm)	S.E. ±	Minimum (cm)	Maximum (cm)	Mean (cm)	S.E. ±	Minimum (cm)	Maximum (cm)	Mean (cm)	S.E. ±	Minimum (cm)	Maximum (cm)	Mean (cm)	S.E. ±
Aspergillus niger (gi:JX112703)	0.0	3.0	1.9^{a}	1.1	1.8	4.00	2.2^{a}	1.0	0.0	4.5	2.5^{b}	1.2	0.5	4.6	2.9^{b}	1.3	2.0	3.0	2.3^{a}	1.1
Aspergillus niger (gi:MG889596	0.0	2.8	1.7^{a}	0.9	0.3	3.4	2.0^{a}	0.9	0.0	4.1	2.2^{b}	1.1	0.2	4.5	2.8^{c}	1.3	0.5	2.0	2.0^{a}	0.8
Aspergillus awamori	0.0	3.1	1.7^{a}	0.9	0.0	3.0	1.9^{a}	0.9	0.3	3.2	2.0^{b}	1.0	0.6	3.7	2.3^{b}	1.1	1.8	2.1	2.0^{b}	0.9
Aspergillus oryzae	0.0	3.0	1.3^{a}	0.8	0.0	2.6	1.5^{a}	0.6	0.0	2.7	1.8^{b}	0.9	0.0	3.6	1.9^{b}	0.8	0.8	3.0	1.8^{b}	1.0
Aspergillus tubingensis	0.0	2.3	1.3^{a}	0.7	0.0	2.5	1.4^{a}	0.5	0.0	2.3	1.7^{a}	0.6	0.0	3.5	1.9^{b}	0.8	0.4	3.0	1.6^{b}	0.7
Penicillium spp.	0.0	2.5	1.8^{a}	0.8	0.0	2.7	1.9^{a}	0.8	0.7	3.8	2.5^{b}	1.3	1.0	4.1	2.8^{b}	1.3	1.6	2.5	1.9^{a}	0.9
Byssochlamys spectabilis	0.0	2.7	1.5^{a}	0.5	0.0	3.5	2.2^{b}	1.0	1.0	3.7	2.5^{c}	1.3	0.0	4.2	3.2^{d}	1.7	1.4	3.0	1.5^{a}	0.5
Trichosporon montevideense	0.0	2.0	1.5^{a}	0.6	0.0	2.8	2.0^{b}	0.9	0.0	3.0	2.5^{c}	1.2	0.0	3.5	2.8^{c}	1.2	1.7	2.2	1.9^{b}	0.8

Means followed by a different letter in the line are significantly different ($p>0.05$).

ZnO NPs; it was completely inhibited after adding 3 and 5% ZnO NPs. *Penicillium* spp. growth decreased by about 11 mm in the control case, while it decreased to about 4 mm after adding ZnO NPs. The amylase activity decreased from 15 mm in the control and to 9 mm after adding 1 and 2% ZnO NPs. 3 and 5% ZnO NPs reduced the amylase activity by about 8 and 4 mm, respectively. *B. spectabilis decreased to* about 15 mm in the control plates, which it decreased to 4 mm in all other treatments. Amylase activity of *B. spectabilis* was 20 mm in the control and antifungal case. It was about 25 mm after adding 1 and 2% ZnO NPs, while it decreased to 19 and 12 mm, respectively after adding 3 and 5% ZnO NPs.

Table 3 reported the effect of adding ZnO NPs on protease enzyme secreted by different fungal species (*Aspergillus* spp.). The fungal growth was about 9 mm in the control, 1, 2 and 3% ZnO NPs samples, while the growth decreased to 8 mm after adding the antifungal drug and 5% ZnO NPs. The protease activity was not detected in *Aspergillus* spp. plates. The growth of *Penicillium* spp. was about 12 mm in the control case, while it was about 4 mm in the case of antifungal drug and all concentrations of ZnO NPs. The protease activity decreased from 16 mm in the control case to 6 mm in 1 and 2% ZnO NPs; while the antifungal, 3 and 5% ZnO NPs completely inhibited it. *B. spectabilis* was about 10 in the control plates, which decreased to 4 mm in all other treatments. Protease activity of *Byssochlamys spectabilis* was 13 mm in the control, while it decreased to 8, 7, 6 and 0 mm in the antifungal drug, 1, 2, 3 and 5% ZnO NPs, respectively.

Table 3 shows the effect of adding ZnO NPs on lipase enzyme secreted by different fungal species (*Aspergillus* spp.). The fungal growth was about 13 mm in the control and 3% samples; while the growth decreased to 12 mm after adding antifungal drug, 1 and 2% samples. The lowest growth recorded was about 11 mm in 5% ZnO NPs concentration. The lipase activity was not detected at all in *Aspergillus* spp. plates. The growth of *Penicillium* spp. was about 14 mm in the control case, while it was about 4 mm in the case of antifungal drugs and all concentrations of ZnO NPs. The lipase activity decreased from 21 mm in the control case to 11, 10, 10, 9 and 6 mm in case of the antifungal drug, 1, 2, 3 and 5% ZnO NPs, respectively. *B. spectabilis* recorded about 16 mm in the control plates, which decreased to 4 mm in all other treatments. Lipase activity of *B. spectabilis* was 22 mm in the control and 20 mm with antifungal case; it was about 18, 18, 17 and 16 mm after adding 1, 2, 3 and 5% ZnO NPs, respectively.

Effect of adding ZnO NPs to different fish types packages on their shelf life

Table 4 and Figures 3 to 10 show the effect of adding different concentrations of ZnO NPs on the shelf life of different fish species samples in comparison with the control samples under refrigeration temperature. In the case of Asian seabass "*L. calcarifer*", bream "*P. pagrus*", rabbitfish "*S. rivulatus*", their shelf life extended from 3 days in the control sample to about 4, 5, 10, and 18 days after adding 1, 2, 3 and 5% ZnO NPs concentrations, respectively. The shelf life of saddle grouper "*Najil P. pessuliferus*" was extended

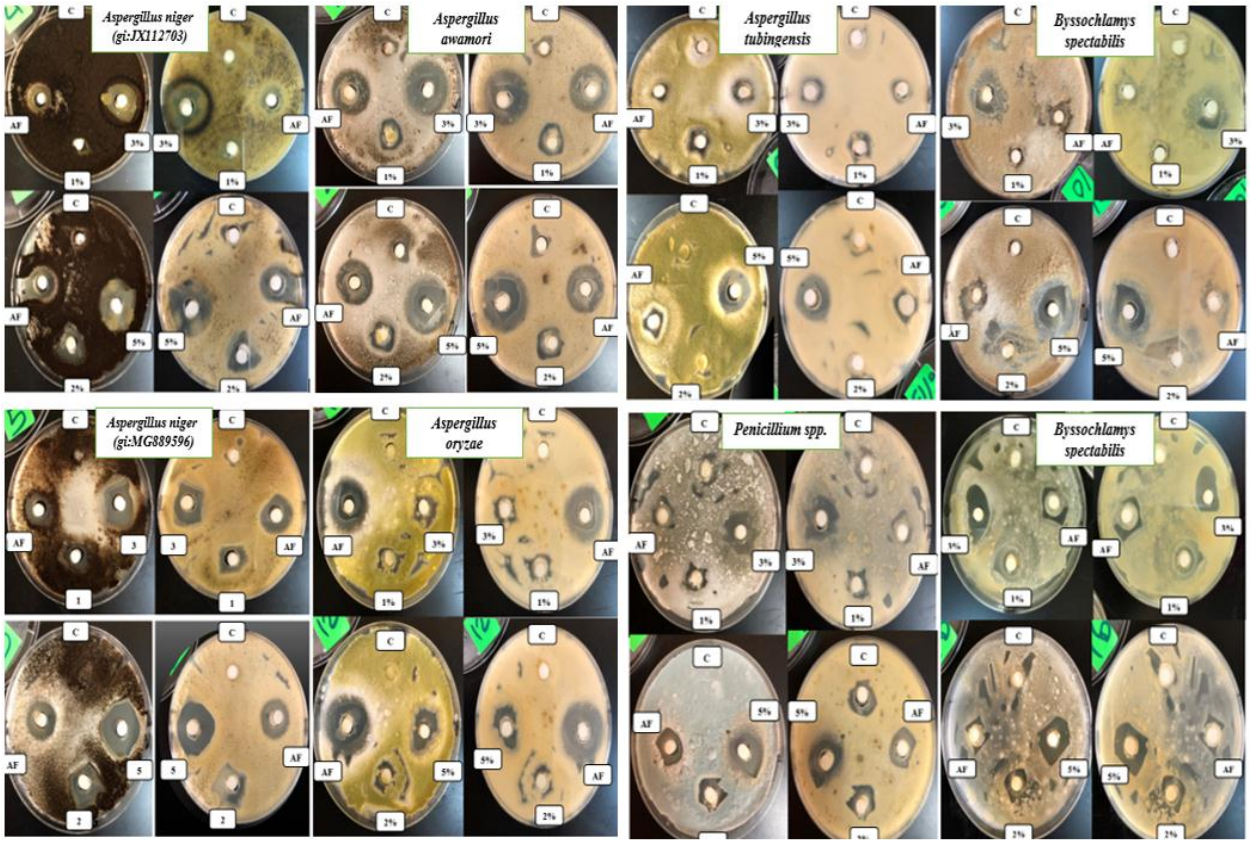

Figure 2. Antifungal effect of different ZnO NPs concentration against isolated fungus. C = Control, AF = Antifungal Concentration, 1% = 1% ZnO NPs Concentration, 2% = 2% ZnO NPs Concentration, 3% = 3% ZnO NPs Concentration, 5% = 5% ZnO NPs Concentration.

to about 2 days in the control and 1% samples, 3 days in 2% ZnO NPs concentration, 9 days and 15 days in 3 and 5% ZnO NPs, respectively.

It was observed that the saddle grouper "*Najil P. pessuliferus*" samples were the least affected by adding different concentrations of ZnO NPs, which got to 2 weeks in 5% concentration. The shelf life of almost all the fish types was affected positively by the addition of different concentrations of ZnO NPs.

DISCUSSION

The different fungus species are arranged as follows: 28% *A. niger* (gi:JX112703), 18.4% *A. oryzae,* 12.3% *A. awamori* and *Penicillium* spp., 9.4% *. tubingensis,* 8.2% *T. montevideense*, 8.0% *A. niger* (gi:MG889596), and 3.4% *B. spectabilis*. Most other studies reported that *A. niger* as the primary spoilage fungi affected different fish species. Park et al. (2014) found about 95.21% of fish fungal spoilage caused mainly by *A. niger*; about 33.3% *A. niger*, which is considered the most predominant fungal isolates (Odu and Ameweiye, 2003). On the other hand, lower results were reported by Samaha et al. (2015) who found fungal fish spoilage as follows: 24% *A. niger* and 48% *Penicillium* spp. Greco et al. (2015) found that *A. niger* predominated (57%) followed by *Penicillium* spp. (12.84%) in fish samples. While, Iqbal and Saleemi (2013) found fungal spoilage of fish in Punjab by *Aspergillus* spp. (78.5%) and *Penicillium* spp. (3.5%). Akwuobu et al. (2019) recorded also that the main fungal genera that contaminated fish sold in Makurdi were *Aspergillus* (28.6%), and *Penicillium* spp. (18.2%).

Aspergillus spp. causes a disease known as "aspergillosis", which appeared as cough, fever, breathlessness, or chest pain. The incidence of infection can be more common between immunosuppressed patients or those who suffer another pulmonary condition. Several species of *Aspergillus* spp., which often contaminate food, are *A. niger*, *A. oryzae*, *A. awamori*, and *A. tubingensis* (Singapurwa et al., 2018; Akwuobu et al., 2019). *Byssochlamys* spp. mostly occurs in compost, air, and different food items. Generally, this fungus accommodates heat above 85°C and microaerophilic condition results in mycotoxins production such as deoxynivalenol and vomitoxin (Casas-Junco et al., 2017).

Polluted aquaculturing has effect on the immunity of fish. This results in the rapid death of fish and makes

Table 3. The mean value of the effect of addition of ZnO NPs on different fungus species enzymes activity.

ZnO nano. Concentration	Amylase Enzyme Activity (mm)						Protease Enzyme Activity (mm)						Lipase Enzyme Activity (mm)					
	Aspergillus spp.		*Penicillium* spp.		*Byssochlamys spectabilis*		*Aspergillus* spp.		*Penicillium* spp.		*Byssochlamys spectabilis*		*Aspergillus* spp.		*Penicillium* spp.		*Byssochlamys spectabilis*	
	G	C.Z	G	C.Z	G	C.Z	G	C.Z	G	C.Z	G	C.Z	G	C.Z	G	C.Z	G	C.Z
Control	14	19	11	15	15	20	9	0.0	12	16	10	13	13	0.0	14	21	16	22
Antifungal	4	8	4	9	4	20	8	0.0	4	0.0	4	8	12	0.0	4	11	4	20
1%	4	7	4	9	4	25	9	0.0	4	6	4	7	12	0.0	4	10	4	18
2%	4	7	4	9	4	25	9	0.0	4	6	4	7	12	0.0	4	10	4	18
3%	4	0.0	4	8	4	19	9	0.0	4	0.0	4	6	13	0.0	4	9	4	17
5%	4	0.0	4	4	4	12	8	0.0	4	0.0	4	0.0	11	0.0	4	6	4	16

Inoculums disc 4 mm; G: Growth, C.Z: Clear zone.

fish get spoiled rapidly after catch. This is due to high fungal and bacterial opportunist including the lower nutritive value of fish caused by stress syndrome from the different aquatic pollutants (Bukola and Zaid, 2015).

ZnO NPs have antimicrobial effect by disintegrating the cell wall of microbes via lysis. The morphology of the micro fungus changes after treatment. ZnO NPs have potent antifungal effect (Shen et al., 2015). The high fungicidal effect of ZnO NPs in this study may be due to their small size (Rajiv et al., 2013; Jeong et al., 2014). ZnO NPs may affect the permeability membrane of the microbial cells, releasing the membrane proteins and lipids. This results in the death of the microbial cell (Padalia and Chanda, 2017). The properties of ZnO NPs used for the development of fungicides have become an urgent issue in medicine and microbial food control (Kairyte et al., 2013). Padalia and Chanda (2017) reported that ZnO NPs have very effective antifungal activity; they have better effect than standard antibiotic amphotericin B. Rajeshkumar (2019) had similar results that fungal inhibition effect correlated inversely with size and concentration of ZnO NPs and concluded that ZnO NPs acted very impressively against the fungal pathogens.

Amylases are the most vital extracellular enzymes which hydrolyze the molecules of starch resulting in diverse products such as dextrin and composed of glucose unit (Gupta et al., 2003). Amylase can be obtained from several fungi. Several studies reported that fungal origin amylases are more stable (Sanghvi et al. 2011). Malaikozhundan et al. (2020) reported almost similar results that ZnO NPs greatly inhibited the microbial amylase activity to about 25 and 25 $\mu g/mL^{-1}$; protease activity was inhibited and lipases activity was inhibited to 25 $\mu g/mL^{-1}$. Lower results were reported by Namasivayam et al. (2016) where microbial enzyme activity was inhibited by metal nanoparticles, which revealed a broad surface plasmon peak presented at 430 nm. Nanoparticles are extremely stable for many months after the reaction. The enzyme was not inhibited at all by the tested concentrations. As in the control samples, there was no significant difference in the enzyme activity ($P>0.05$), which revealed 6.02, 6.18, 6.23, 6.53, 6.88 µ/ml and 4.52, 4.50, 4.68, 4.72 µ/ml, respectively. Also, Verma and Verma, (2018) recorded amylase produced by the fungi as follows: *Penicillium* spp. had the highest amylase production 1(0.93 cm) followed by *Aspergillus* spp. (0.6 cm); Kathiresan and Manivannan (2006) recorded *Penicillium* spp. produced maximum amylase (136 U/ml). Sharma and Shukla (2008) reported that maximum amylase was produced by *Aspergillus* spp. (185 U/ml).

Freshness is one of the parameters used to judge the quality of fish and can be determined by using the sensory analysis method. Sensory analysis is simple, fast, and provides immediate quality information about the tested fish products. The sensory characteristics of fish are obvious to the fish consumers and are essential for the consumption of fish and its products (Reineccius, 1991). Results observed that the saddle grouper "*Najil P. pessuliferus*" samples were the least affected after adding ZnO NPs (different concentrations); it got to 2 weeks after adding 5% ZnO NPs. The shelf life of almost all the types of fish was affected positively by the addition of different concentrations of ZnO NPs.

Researchers reported that ZnO NPs molecules must penetrate or be in contact with microbial cells to perform their inhibitory activity (Mohd et al., 2019). Similar results of ZnO NPs effect on the cell

Table 4. Effect of addition of ZnO NPs to different fish types package on their shelf life.

Types of fish	Control (day)	1% (day)	2% (day)	3% (day)	5% (day)
Asian seabass "*Lates calcarifer*"	3	4	5	10	18
Bream "*Pagrus pagrus*"	3	4	5	10	18
Rabbitfish "*Siganus rivulatus*"	3	4	5	10	18
Spangled emperor "*Lethrinus nebulosus*"	2	3	5	10	18
Gilthead seabream "*Sparus aurata*"	2	3	4	10	18
Mackerel fish "*Scomberomorous commerson*"	2	3	4	10	18
Red mullet "*Mullussurmuletus*"	2	3	4	10	18
Saddle Grouper "*Najil P. pessuliferus*"	2	2	3	9	15

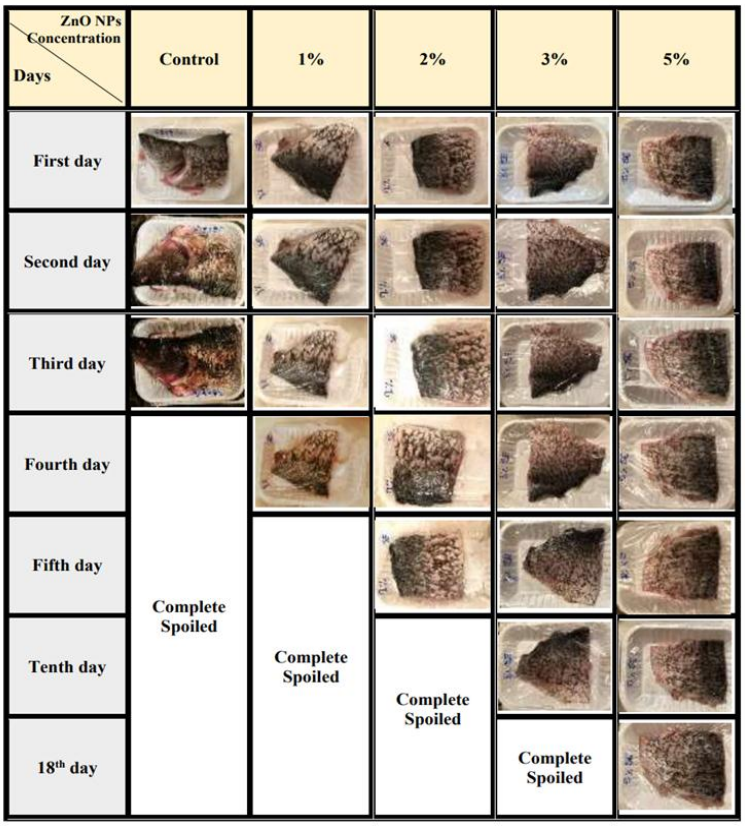

Figure 3. Effect of addition of ZnO NPs on "Asian seabass" fish sensory characters.

wall of microbes were given by Shawky et al. (2014). They noticed that the antimicrobial effect of ZnO NPs occurred in 2 ways: firstly, H_2O_2 was formed on ZnO NPs surface due to the hydrogen bond between the hydroxyl group of fungi cellulose molecules and the atom of oxygen of ZnO NPs, resulting in the inhibition of the fungal growth; secondly Zn^{2+} was released leading to cell membrane damage and interaction with intracellular contents (Moraru et al., 2003).

Similar antifungal activities of ZnO NPs inhibited different fungal growth. They increased with higher contraction of ZnO NPs, especially 200 and 300 ug/ml; they had about 7 to 15 mm inhibition diameter (Hassan et al., 2014). This inhibition effect was clearer against *Aspergillus* spp. (1.013296 µg/ml) and fluconazole which were 0.001-0.56 and 0.062-128 µg/ml, respectively. ZnO NPs changed the microbial cell structure of the fungi including the cell membrane, leading to leakage of the cytoplasm and distribution of the fungal cells. ZnO NPs can inhibit conidial development and damage conidiophores or hyphae (He et al., 2011).

The daily dietary intake of zinc for adults is about

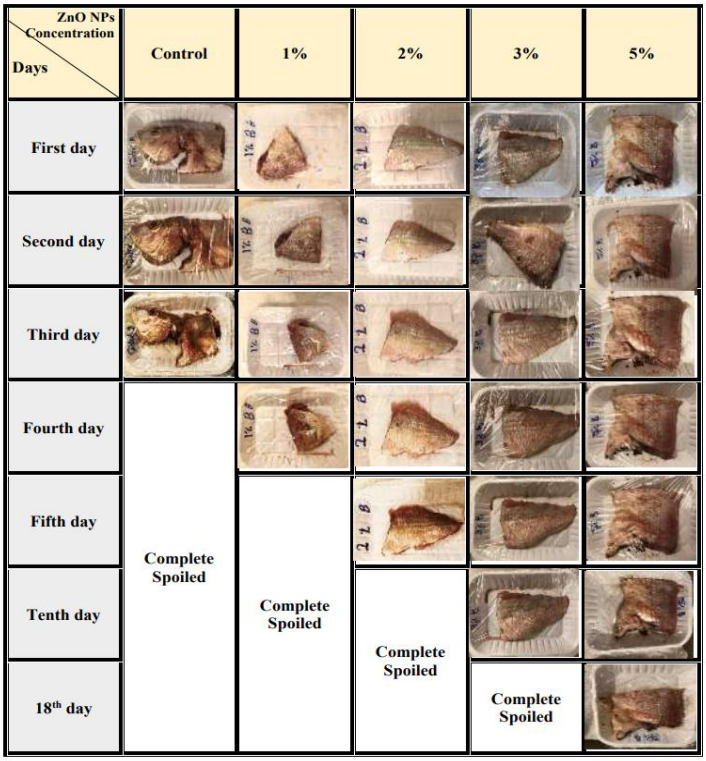

Figure 4. Effect of addition of ZnO NPs on “Bream” fish sensory characters.

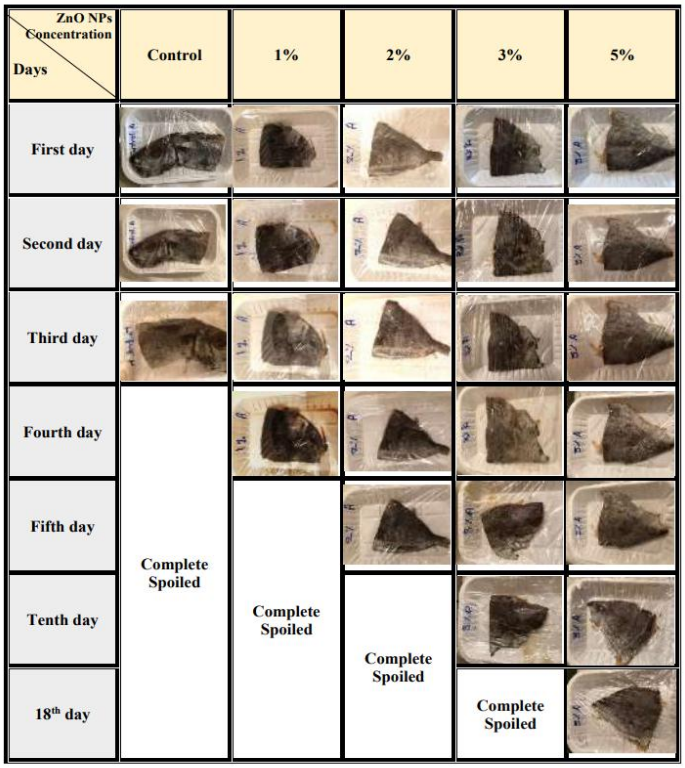

Figure 5. Effect of addition of ZnO NPs on “Rabbitfish” fish sensory characters.

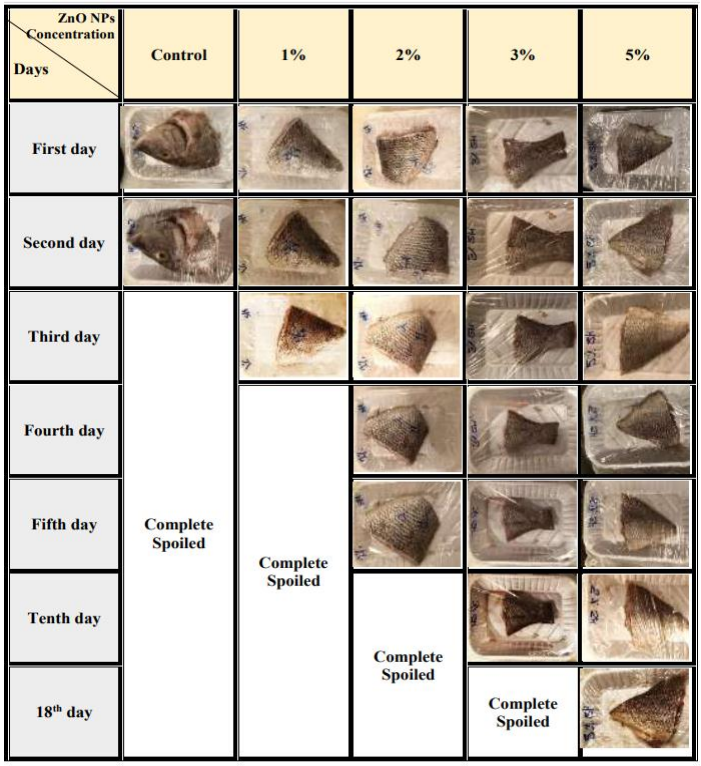

Figure 6. Effect of addition of ZnO NPs on "Spangled emperor" fish sensory characters.

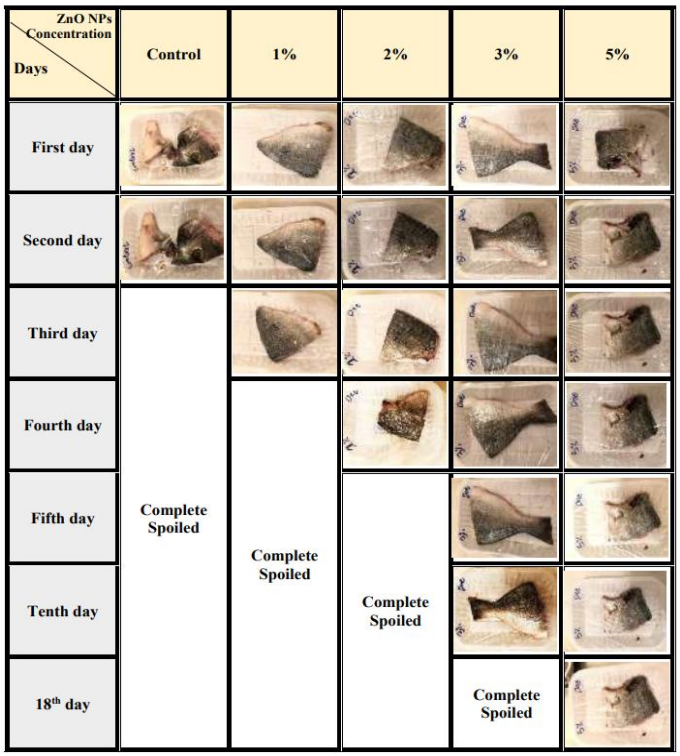

Figure 7. Effect of addition of ZnO NPs on "Gilthead Seabream" fish sensory characters.

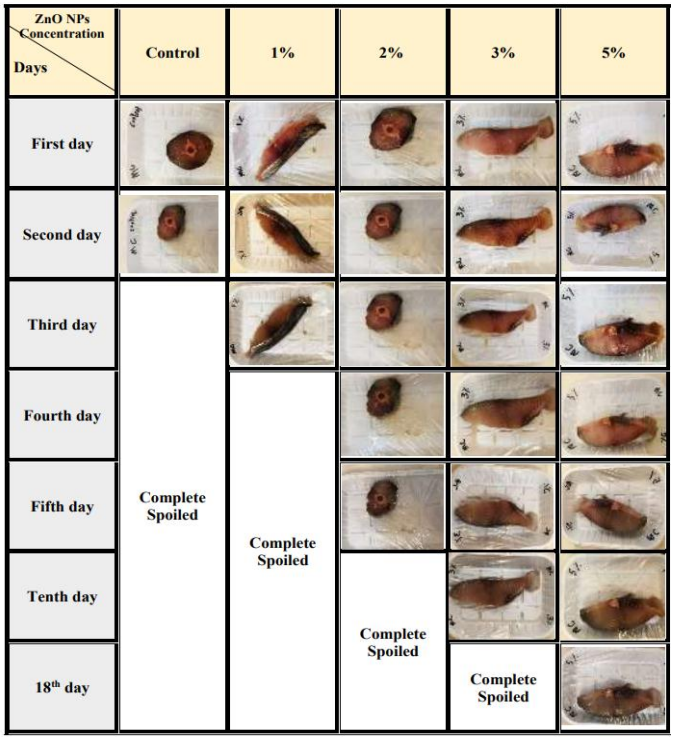

Figure 8. Effect of addition of ZnO NPs on “Mackerel” fish sensory characters.

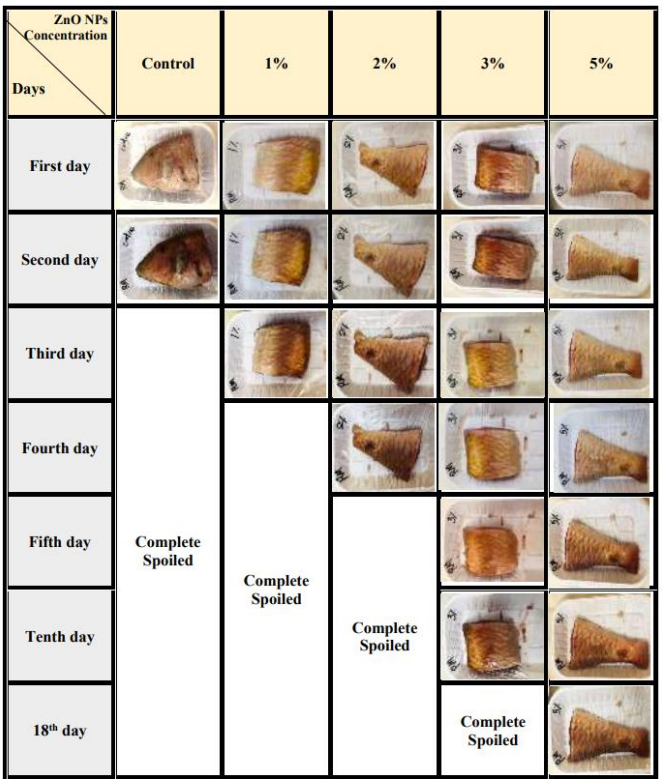

Figure 9. Effect of addition of ZnO NPs on “Red mullet” fish sensory characters.

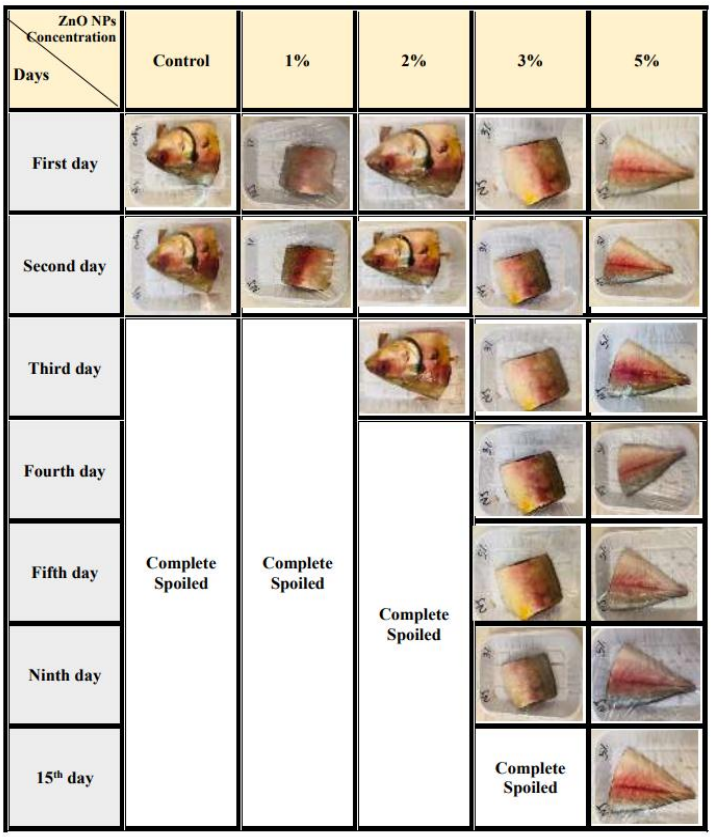

Figure 10. Effect of addition of ZnO NPs on "Saddle Grouper" fish sensory characters.

40 mg, which is equivalent to 143 ml of liquid egg daily intake of 0.28 mg of ZnO per ml (Hassan et al., 2014). According to the National Research Council, the recommended dietary allowances (RDA) for humans are about 15,000 and 12,000 mg/different for healthy men and women (Yilmaz and Aksoy, 2006). Sensory attributes of fish including their juiciness, appearance or tenderness, odor, flavor, aftertaste, and acceptability scores were significantly decreased ($P<0.05$) with prolonged storage time (Sallam, 2007).

Conclusion

It is concluded that there is a gradual increase in the fungal growth with increased ZnO NPs concentration in all tested fish species, especially at 3 and 5% concentration. It is recommended to use 3% concentration of ZnO NPs to improve the safety of fish and prolong their shelf life up to 15 days.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

REFERENCES

Akwuobu CA, Antiev WS, Ofukwu RP (2019). Fungal Contaminants of Smoke-Dried Fish Sold in Open Markets in Makurdi, Benue State, North-Central Nigeria. Food and Nutrition Sciences 10(3):290-297.

Ali OG (1992). Formation of proteases by *Aspergillus fumigatus* and *Pencillium* sp. King Saud University–Science 4(2):127-136.

Al-Naamani L, Dobretsov S, Dutta J (2016). Chitosan-zinc oxide nanoparticle composite coating for active food packaging applications. Innovative Food Science & Emerging Technologies 38:231-237.

Ayalew A, Fufa A (2018). Maintenance of Fish Health in Aquaculture: Review of Epidemiological Approaches for Prevention and Control of Infectious Disease of Fish. Hindawi Veterinary Medicine International 1-10.

Bradley EL, Castle L, Chaudhry Q (2011). Applications of nanomaterials in food packaging with a consideration of opportunities for developing countries. Trends in Food Science & Technology 22(11):604-610.

Bukola D, Zaid A (2015). Consequences of Anthropogenic Activities on Fish and the Aquatic Environment. Poultry, Fisheries & Wildlife Sciences 3(2):1000126-1000138

Cannas D, Loi E, Serra M, Firinu D, Valera P, Zavattari P (2020). Relevance of essential trace elements in nutrition and drinking water for human health and autoimmune disease risk. Nutrients 12(7):2074.

Cao F, Yang Q, Shao CL, Kong CJ, Zheng JJ, Liu YF, Wang CY (2015). Bioactive 7-oxabicyclic [6.3. 0] lactam and 12-membered macrolides from a gorgonian-derived *Cladosporium* sp. fungus. Marine Drugs 13(7):4171-4178.

Casas-Junco PP, Ragazzo-Sánchez JA, de Jesus Ascencio-Valle F, Calderón-Santoyo M (2017). Determination of potentially mycotoxigenic fungi in coffee (*Coffea arabica* L.) from Nayarit. Food

Science and Biotechnology 27(3):891-898.
Chavan BR, Yakupitiyaga A, Kumar S (2011). Drying performance, quality charactersitics and financial evaluation of Indian mackerel dried by a solar tunnel dryer. Science & Technology Asia 16(2):11-25.
CODEX A (2009). Code of Practice for fish and fishery products, First edition, Rome: World Health Organization Food and Agriculture Organization of United Nations Rome (FAO).
Crona B, Wassénius E, Troell M, Barclay K, Mallory T, Fabinyi M, Zhang W, Lam V, Cao L, Henriksson P (2020). China at a crossroads: an analysis of China's changing seafood production and consumption. One Earth 3(1):32-44.
Derrick S (2009). Cured, smoked and dried fish In R. Fernandes (Ed.), microbiology handbook fish and seafood (pp. 93-121): Leatherhead Publishing, a division of Leatherhead Food International Ltd Randalls Road, Leatherhead, Surrey KT22 7RY, UK.
Duncan TV (2011). Applications of nanotechnology in food packaging and food safety: barrier materials, antimicrobials and sensors. Journal of Colloid and Interface Science 363(1):1-24.
Ebrahimiasl S, Rajabpour A (2015). Synthesis and characterization of novel bactericidal Cu/HPMC BNCs using chemical reduction method for food packaging. Journal of Food Science and Technology 52(9):5982-5988.
Fraternale D, Giamperi L, Ricci D (2003). Chemical composition and antifungal activity of essential oil obtained from in vitro plants of Thymus mastichina L. Journal of Essential Oil Research 15(4):278-281.
Greco M, Pardo A, Pose G (2015). Mycotoxigenic fungi and natural co-occurrence of mycotoxins in rainbow trout (*Oncorhynchus mykiss*) feeds. Toxins 7(11):4595-4609.
Gupta R, Gigras P, Mohapatra H, Goswami VK, Chauhan B (2003). Microbial α-amylases: a biotechnological perspective. Process Biochemistry 38(11):1599-1616.
Hassan A, Oraby N, Mohamed A, Mahmoud H (2014). The possibility of using Zinc Oxide nanoparticles in controlling some fungal and bacterial strains isolated from buffaloes. Egyptian Journal of Applied Science 29(3):58-83.
He L, Liu Y, Mustapha A, Lin M (2011). Antifungal activity of zinc oxide nanoparticles against *Botrytis cinerea* and *Penicillium expansum*. Microbiological Research 166(3):207-215.
Iqbal Z, Saleemi S (2013). Isolation of pathogenic fungi from a freshwater commercial fish, Catla catla (Hamliton). Science International (Lahore) 25(4):851-855.
Javadi M, Ghanbary M, Tazick Z (2012). Isolation and molecular identification of soil inhabitant Penicillia. Annals of Biological Research 3(12):5758-5761.
Javed M, Ahmad I, Usmani N, Ahmad M. (2016). Studies on biomarkers of oxidative stress and associated genotoxicity and histopathology in Channa punctatus from heavy metal polluted canal. Chemosphere 151:210-219.
Jeong Y, Lim D, Choi J (2014). Assessment of size-dependent antimicrobial and cytotoxic properties of silver nanoparticles. Advances in Materials Science and Engineering 13:100-110.
Kairyte K, Kadys A, Luksiene Z (2013). Antibacterial and antifungal activity of photoactivated ZnO nanoparticles in suspension. Journal of Photochemistry and Photobiology B: Biology 128:78-84.
Kathiresan K, Manivannan S (2006). Amylase production by *Penicillium fellutanum* isolated from mangrove rhizosphere soil. African Journal of Biotechnology 5(10):829-832.
Khan AQ, Aldosari F, Hussain SM (2018). Fish consumption behavior and fish farming attitude in Kingdom of Saudi Arabia (KSA). Journal of the Saudi Society of Agricultural Sciences 17(2)195-199.
Koh L, Tan T, Chou L, Goh N (2000). Fungi associated with gorgonians in Singapore. Proceedings of the Ninth International Coral Reef Symposium, Bali, Indonesia 23-27(1):521-526.
Magwaza NM, Nxumalo EN, Mamba BB, Msagati TM (2017). The Occurrence and Diversity of Waterborne Fungi in African Aquatic Systems: Their Impact on Water Quality and Human Health. International Journal of Environmental Research and Public Health 14(5):546.
Malaikozhundan B, Vinodhini J, Kalanjiam M, Vinotha V, Palanisamy S, Vijayakumar S, Mariyappan A (2020). High synergistic antibacterial, antibiofilm, antidiabetic and antimetabolic activity of Withania somnifera leaf extract-assisted zinc oxide nanoparticle. Bioprocess and Biosystems Engineering 43(9):1533-1547.
Mohd H, Mohamad R, Zaidan UH, Abdul Rahman N (2019). Microbial synthesis of zinc oxide nanoparticles and their potential application as an antimicrobial agent and a feed supplement in animal industry: a review. Journal of Animal Science and Biotechnology 10(1):1-22.
Namasivayam S, Aroma R, Manikanta M, Gopinath P, Francis A (2016). Evaluation of enzyme activity inhibition of biogenic silver nanoparticles against microbial extracellular enzymes. Journal of Pharm Tech Research 9(2):40-47.
Ngo H, Tran L, Nguyen D, Nguyen T, Le Th, Gao Y (2021). Metal Pollution and Bioaccumulation in the Nhue-Day River Basin, Vietnam: Potential Ecological and Human Health Risks. International Journal of Environmental Research and Public Health 18(24):13425.
Odu NN, Ameweiye NB (2013). Microbiological Quality of Street Vended-Ready-To-Eat "Bole" Fish In Port Harcourt Metropoplis. New York Science Journal 6(2):92-101.
Padalia H, Chanda S (2017). Characterization, antifungal and cytotoxic evaluation of green synthesized zinc oxide nanoparticles using *Ziziphus nummularia* leaf extract. Artificial Cells, Nanomedicine, and Biotechnology 45(8):1751-1761.
Park SY, Lee NY, Kim SH, Cho JI, Lee HJ, Ha SD (2014). Effect of ultraviolet radiation on the reduction of major food spoilage molds and sensory quality of the surface of dried filefish (*Stephanolepis cirrhifer*) fillets. Food Research International 62:1108-1112.
Paul SK, Dutta H, Sarkar S, Sethi LN, Ghosh SK (2019). Nanosized Zinc Oxide: Super-Functionalities, Present Scenario of Application, Safety Issues, and Future Prospects in Food Processing and Allied Industries. Food Reviews International 35(6):505-535.
Qasim C (2011). Food Applications of Nanotechnologies: An Overview of Opportunities and Challenges for Developing Countries. Trends in Food Science & Technology 22(11):595-603.
Rahman MA, Rahman MH, Yeasmin S, Asif A, Mridha D (2017). Identification of Causative Agent for Fungal Infection and Effect of Disinfectants on Hatching and Survival Rate of Bata (*Labeo. Bata*) Larvae. Advances in Plants & Agriculture Research 7:00264.
Rajeshkumar S (2019). Antifungal Impact of Nanoparticles Against Different Plant Pathogenic Fungi. Nanomaterials in Plants, Algae and Microorganisms 197-217.
Rajiv P, Rajeshwari S, Venckatesh R (2013). Bio-Fabrication of zinc oxide nanoparticles using leaf extract of *Parthenium hysterophorus* L. and its size-dependent antifungal activity against plant fungal pathogens. Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy 112:384-387.
Reineccius G (1991). Off-flavors in foods. Critical Reviews in Food Science & Nutrition 29(6):381-402.
Rico-Munoz E, Samson R, Houbraken J (2018). Mould spoilage of foods and beverages: Using the right methodology. Food Microbiology 81:51-62.
Ross D (1976). Invertase and amylase activities in ryegrass and white clover plants and their relationships with activities in soils under pasture. Soil Biology and Biochemistry 8(5):351-356.
Sallam KI (2007). Chemical, sensory and shelf life evaluation of sliced salmon treated with salts of organic acids. Food Chemistry 101(2):592-600.
Samad MA, Razak CA, Salleh AB, Yunus WW, Ampon K, Basri M (1989). A plate assay for primary screening of lipase activity. Journal of Microbiological Methods 9(1):51-56.
Samaha IA, Amer AA, Abd-Elshahid YS, El-bialy SM (2015). Mycological Evaluation of Imported Frozen Fish. Alexandria Journal for Veterinary Sciences 5:40-45.
Sanghvi GV, Koyani RD, Rajput KS (2011). Isolation, optimization, and partial purification of amylase from *Chrysosporium asperatum* by submerged fermentation. Journal of Microbiology and Biotechnology 21(5):470-476.
Sharma D, Shukla A (2008). Starch hydrolysis and alpha-amylase activity of Aspergillus and Chaetomium. Asian Journal of Biochemistry 3(5):284-289.
Shawky NM, Hassan AA, ElAhl RM, Mahmoud HH (2014). Evaluation of the antimicrobial effect of zinc oxide nanoparticles on *Listeria monocytogenes* and *Candida albicans* isolated from infected Egyptian

buffalo suffering from abortion. 2nd Scientific Conference of Scientific Association of Animal Health Research Institute 2(4):142-157.
Shen Z, Chen Z, Hou Z, Li T, Lu X (2015). Ecotoxicological effect of zinc oxide nanoparticles on soil microorganisms. Frontiers of Environmental Science & Engineering 9(5):912-918.
Singapurwa NS, Suprapta DN, Gunam IW, Wirya IG, Khalimi K (2018). Identification of Contaminant Fungi on Pedetan, a Dry Fish Product of Lemuru (*Sardinella lemuru*). Journal of Biology, Agriculture and Healthcare 8(6):73-82.
SPSS 16 (2007). SPSS for windows Release 16.0.0, Standard Version, Copyright SPSS Inc., 1989-2007, All rights reserved, Copyright® SPSS Inc. Statistical Package for Social Science, SPSS for windows Release 16.0.0, Standard Version.
Stoimenov PK, Klinger RL, Marchin GL, Klabunde KJ (2002). Metal oxide nanoparticles as bactericidal agents. Langmuir 18(17):6679-6686.
Swain P, Nayak S, Sasmal A, Behera T, Barik S, Swain S, Jayasankar P (2014). Antimicrobial activity of metal based nanoparticles against microbes associated with diseases in aquaculture. World Journal of Microbiology and Biotechnology 30(9):2491-2502.
Tacon AJ (2020). Trends in global aquaculture and aquafeed production: 2000–2017. Reviews in Fisheries Science & Aquaculture 28(1):43-56.
Verma P, Verma R (2018). Qualitative estimation of amylase enzyme activity of fungal species isolated from iron ore mined overburden soil. Journal of the Society for Tropical Plant Research 5(3):396-404.
White P (2013). Environmental consequences of poor feed quality and feed management. In M.R. Hasan and M.B. New, eds. On-farm feeding and feed management in aquaculture. FAO Fisheries and Aquaculture Technical Paper No. 583. Rome, FAO. pp. 553-564.
Yilmaz D, Aksoy A (2006). Heavy metal levels in vegetables in Turkey are within safe limits for Cu, Zn, Ni and exceeded for Cd and Pb. Journal of Food Quality 29(3):252-265.
Zaitz C, Ruiz LB, Souza VM (2004). Atlas de micologia médica: diagnóstico laboratorial. In Atlas de micologia médica: diagnóstico laboratorial (pp. 167-167). Medsi, 2º ed. Rio de Janeiro.
Zhang L, Jiang Y, Ding Y, Povey M, York D (2007). Investigation into the antibacterial behaviour of suspensions of ZnO nanoparticles (ZnO nanofluids). Journal of Nanoparticle Research 9(3):479-489.

Related Journals: