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ISOLATION AND IDENTIFICATION OF LACTIC ACID BACTERIA STRAINS AND THEIR EFFECTS ON THE FERMENTATION QUALITY OF ELEPHANT GRASS (*PENNISETUM PURPUREUS*) SILAGE

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ABSTRACT

In Nigeria, animal agriculture serves very paramount diversified role in animal protein food source, farm power, farm manure as well as ensuring social status-quo and enriching livelihood with other products. Most of the natural grasses are deficient in essential nutrient due to poor soils, yet, there is growing demand for dairy products

Introduction

Nigeria is reported as a major hub of animal product consumption in West Africa. She is one of the largest livestock-raising countries in the region. Meeting the ever-increasing domestic demand and access to these flourishing markets are major economic stakes for Nigeria and for the neighbouring countries that raise livestock (Catley *et al*., 2012)**.** Like humans, livestock animals need a balanced diet containing all the necessary nutrients, fluids, minerals, and vitamins. Proper nutrition guarantees animals the vigour to grow, develop,

globally. Some lactic acid bacteria (LAB) are naturally able to produce metabolites that could enrich silage during fermentation thereby improving their qualities as animal feeds stock under intensive system. This research was conducted to isolate, screen and identify some LAB cultures from fermented silage for fermentation of fresh Elephant grass (*Pennisetum purpureum*). The lactic acid bacteria were isolated and identified from silage using MRS isolation media in a pour plate method and characterized using biochemical and molecular technique. The confirmed pure cultures (*L. plantarum* (LB09), *L. casei* (LB37) and *L. lactis* (LB75) were used to ferment fresh elephant grass for sixty-days after which the proximate compositions of the fermented product (silage) were determined and compared with LAB-free silage. The results show that Dry matter increased from 18.42 to 26.12, so also the crude protein (5.67 to 7.32), NPF (59.25 to 62.12), ADF (57.43 to 61.54) respectively. The moisture content and the pH decreased favourably from 40% to 35% and from 4.7 to 3.6 respectively. Implementation of intensive and subsequent improvement of dairy production system in Nigeria are promising and achievable if programmed feedstock production such as controlled silage fermentation are implemented.

Keywords*: Dairy, Silage, Fermentation, enrichment, Metabolite and Lactic Acid Bacteria.*

and reproduce, and strong immunity to fight off infections. This basic knowledge leads to more profitable and sustainable animal production in Nigeria's post-Covid economic revitalization (Agboola and Balcilar, 2012).

Forages are essential for the successful operation of animal production systems. This is more relevant to all ruminants which are heavily dependent upon forages for their health and production in a cost-effective and sustainable manner. They are an economical source of nutrients for animal production; they also help conserve the soil integrity, water supply and air quality. However, with the increasing

global human population and urbanisation, the sustainability of forage-based animal production systems is sometimes questioned due to the interrelationship between animal production, security and the environment (Boland *et al*., 2013).

Grasses contain crude fibres, crude protein and some minerals. Legumes are particularly rich in proteins and minerals. Root crops are high in starch and sugar and low in fibre, making them easy to digest. The fibre content of most fodder crops consists of cellulose, a complex carbohydrate polysaccharide that is indigestible for humans, but which is a good source of energy for animals, and particularly ruminants (Cai, 2004). These natural resources are excellent especially when they are further broken down by some microorganisms to release the nutrients in a simpler and easier to absorb forms (Chung *et al*., 2014).

For long, Lactic acid bacteria (LAB) have been playing important roles in food, agricultural, and clinical applications. In recent time, feed preparations especially for intensive animal production have gained interest. Feed fermentation is a complex process that integrates knowledge from nutrition, physiology, immunology, microbiology, biochemistry, industry design, ecology, economy, and bioinformatics. Advanced technologies have increased the knowledge about the various principles underlining feed formulations from different microbial consortia. However, the use of techniques for feed fermentation depends on the nutritional requirements and digestive physiology of animals, the nutritive value of feedstuffs, fermentation characteristics of the microorganisms added as the starter culture, and actual situations on individual farms. Depending on needs, such as the age, lactation and season, the nutritional requirements of the particular animal are determined in order to ascertain the actual nutritive values of fermented feedstuffs to be given (Chen *et al*., 2014).

Ensiling as a traditional method for preserving fresh forage provides animals with high nutritious feeds throughout the year. During the fermentation process, lactic acid bacteria (LAB) multiply under anaerobic condition, and could ferment the substrate (Fodder) into lactic acid (LA) and other acids (Mugnai *et al*., 2017). To obtain high-quality feeds for livestock, LAB is often employed as silage inoculants. Currently, most of

LAB inoculants for ensiling were isolated from plant-derived materials, including fresh forage, silage, and so on (Mugnai *et al*., 2017).

Lactic Acid Bacteria constitutes a group of Gram-positive bacteria that share similar morphologic, metabolic, and physiologic characteristics. They are non-spore-forming rods and cocci that ferment carbohydrates forming lactic acid as the major end-product. Depending on the metabolic pathways they use to ferment carbohydrates and the resulting end-products, they are divided into two major groups: homofermentative or heterofermentative. LAB is capable of producing inhibitory substances other than organic acids with inhibitory activity to different microorganisms. Some lactic acid bacteria produce metabolites that give reductions of food-borne pathogens and spoilage microorganisms in farm animal production system (Babatunde and Qaim, 2010).

The use of controlled fermentations using known starter culture microorganisms is one method receiving considerable recent attention as a natural alternative to enhancing animal productivity and improving product safety via protective effects of the LAB metabolites such as hydrogen peroxide, benzoic acid, diacetyl, mevalonolactone, and reuterin (β-hydroxypropionaldehyde) that can inhibit food-borne pathogens and silage spoilage microorganisms (Alirol *et al*., 2011).

Plant-derived LAB may provide a new approach for the development of silage inoculant. This is because, microbial fermentation can produce array of end products and can change many nutritive aspects of forage. Expectedly, high-quality silage should be void of undesirable compounds that could negatively affect animal performance, the environment, or net farm income. Currently, animal production based on extensive nomadic system is saddled with many challenges including low feed nutrient intake from the natural grasses, low amount of rain for the growth of the pastures, insecurity and Fulani-farmers clash. Hence, in the current effort to improve Nigeria economy through animal production, locally available feed resources may be used for preparing fermented feed ration and improving livestock raising methods to promote the revitalization of local animal husbandry and improve the livelihoods of local people of Nigeria (Boland *et al*., 2013).

The aim of the present study was to isolate, screen and identify some LAB cultures from fermented silage, use the pure cultures for a controlled fermentation of fresh Elephant grass (*Pennisetum purpureum*) and subsequently determine their effect on the fermentation by proximate qualities determination.

Methods and Methodology

Isolation of Lactic Acid Bacteria

One (1 g) gram of fermented silage was mixed with 9ml of sterile peptone water and then serially diluted to 10^{-6}. Then 10^{-2} and 10^{-4} dilutions were Pour-plated on MRS agar medium and incubated at 35°C for 48 h under anaerobic conditions to isolate and purify LAB, respectively. All isolated strains were cultivated on MRS agar medium and their morphological characteristic and growth capacities were studied and documented (Badis *et al.*, 2004).

Screening for Acid Production Capacity

The isolated Lactic Acid Cultures were introduced into sterile MRS broth and incubated for 48 hours respectively. Hand-held pH meter was used to monitor acid production abilities. The LAB strains with fast growth capacity and high acid-producing ability were tested for their physiological and biochemical characteristics (Bourdichon *et al*., 2012).

Molecular Confirmation of Isolates

PCR technique (16S rRNA gene sequencing) was employed to identify the screened strains (Amaral *et al*., 2020).

Out of all the LAB strains were isolated from the silage material and *Lactobacillus plantarum* (LB09), *Lactobacillus casei* (LB37) and *Lactobacillus lactis* (LB75) were used for further studies.

Silage Preparation for Fermentation

Elephant grass (*Pennisetum purpureum*) forage was harvested in early bloom stage of second cutting from an abandoned farmland in Giri, FCT, Abuja. The plant materials were wilted to about 70% moisture content and chopped into an average length of about 10 mm. The chopped material was prepared and packed into plastic film bags, treated with the

Lactic Acid Bacteria isolates (*Lactobacillus plantarum* (LB09), *Lactobacillus casei* (LB37) and *Lactobacillus lactis* (LB75) at 10^6 CFU/g of silage fresh matter respectively. The bags per treatment were performed in triplicate, sealed and stored at room temperature for 100 days (Nascimento, *et al*., 2019).

Determination of Bacteria (LAB) Population

Sample (1g) of the fermented silage was mixed 9mL sterile peptone water and serially diluted to 10^{-4}. Then, 0.1 ml of the chosen dilutions (10^{-1}, 10^{-3}, and 10^{-5}) was spread on the MRS agar respectively. After incubation for 48 hours at 35°C, the number of LAB was enumerated (Zhangm *et al*., 2009).

Determination of Fermentation Quality and Chemical Compositions

The total dry matter contents of spontaneous and controlled fermented forages were determined by drying the sample in an air oven at 65 °C for 48 h. The dried samples were ground to a 1 mm screen by a laboratory mill grinder. Crude protein (CP) was analyzed using a Kjeldahl nitrogen analyzer (Kjeltec 2300 Auto-Analyzer, Sweden) while the crude fat (EE) was determined by an extraction method. Crude ash content (Ash) was detected in an ash furnace by burning a sample at 550 °C for 4 h. Crude fiber (CF), neutral detergent fiber (NDF) and acid detergent fiber (ADF) contents were measured by an A220 Fiber Analyzer (ANKOM Technology Corp., USA). Water soluble carbohydrate (WSC) was determined using the thracenone-sulphuric acid method. Ten grams of each fermented sample was mixed with 90 mL of distilled water, filtered and the pH of the filtrate was taken respectively (Arunachalam *et al*., 2011).

Statistical Analysis

All the statistical analyses were performed using SAS 9.0 software (SAS Institute, Cary, NC, United States, 2002) and the results represented by their mean values. Duncan's multiple range method was used to determine the significant difference between means. The level of statistical significance was declared at $P < 0.05$ and $P < 0.01$.

Results

Table 1: Biochemical Characterization of the Isolated LAB Strains

Isolate	pH (Epv)	Grm Rxt	Cat.	Ferm. type	Growth @45^{0}C	NaCl (6.5%)
LB02	4.02	+	–	Htf	+	+
LB05	4.31	+	–	Htf	+	+
LB09	**3.75**	+	–	Hmf	+	+
LB21	4.42	+	–	Hmf	-	+
LB37	**3.68**	+	-	Hmf	+	+
LB46	4.08	+	–	Htf	+	+
LB71	4.13	+	–	HTf	-	+
LB75	**3.78**	+	–	Hmf	+	+
LB93	4.35	+	–	Htf	-	+
LB97	4.31	+	–	Hmf	+	+

Key: Epv=End point value; - = Negative; + = positive; Hmf = Homofermentative; Htf = Heterofermentative; Cat. = Catalase; Grm Rxt= Grams' Reactions.

Table 2: Sugar Fermentation profile of Isolates

Isolate	Ara.	Cell.	Lac.	Man.	Mel.	Sal.	Sor.	Suc.	Raff.	Treh.
LB09	-	+	+	+	+	+	+	+	+	+
LB37	-	+	+	+	+	+	+	+	+	+
LB75	-	+	+	+	+	+	+	+	+	+

Key: - = Negative; + = positive

Table 3: BLAST Alignment of 16SrDNA sequence of LAB Isolates

Isolate	Related Spp.	Acc. No.	Similarity (%)
LB09	*L. plantarum*	HM448901	100
LB37	*L. casei rhamnosus*	CP053619	84
LB75	*L. Lactis*	CP018215	71

Identification and Screening of LAB

Table 1 shows the biochemical characterization of the LAB strains isolated from silage.

One Hundred and Forty-nine strains of LAB were isolated from silages. The basic characteristics of screened LAB strains are shown. They were Gram-positive and Catalase-negative, homofermentative (Htf) or heterofermentative (Hmf) LAB, low pH tolerant, tolerating salt (MRS with 6.5% NaCl concentrations.

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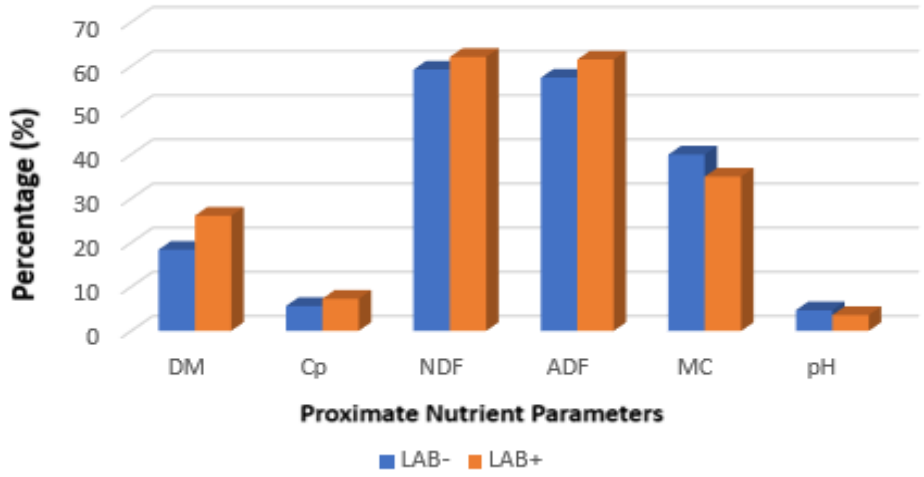
Table 2 shows the sugar fermentation profiles of the LAB isolates on which bases the isolates were screened to ten (10) strains. They were further screened to three (3) strains (LB09, LB37 & LB75) by their high growth and acid-producing rates (3.75, 3.68 & 3.78) respectively.
Table 3 shows the BLAST Alignment of 16SrDNA sequence used to confirm the identities of the respective LAB Isolates. The 16S rRNA sequences were compared using the NCBI database and the results showed that the similarities between all sequences obtained here with the known 16S rRNA gene sequences in the database were 70.0–100.0%. *L. plantarum*, *L. casei* and *L. lactis* were confirmed with identification percentage of 100, 84 and 71 respectively.

Table 4: Bacteria (LAB) Population Dynamics during Fermentation

	Fermentation Time (Days)					
Isolate	10	20	40	60	80	100
LB09	$1.4x10^6$	$2.5x10^7$	$2.1x10^8$	$3.4x10^8$	$3.7x10^8$	$3.9x10^8$
LB37	$2.3x10^6$	$2.9x10^8$	$3.1x10^8$	$5.3x10^8$	$2.1x10^8$	$1.1x10^7$
LB75	$4.8x10^6$	$5.6x10^7$	$9.1x10^8$	$2.2x10^9$	$5.3x10^8$	$4.6x10^8$

Table 4 shows the Bacteria (LAB) population dynamics during fermentation. The number of bacteria increased up to 60 days when they seemed to stabilise. Changes recorded were LB09 ($1.4x10^6$ to $3.4x10^8$), LB37 ($2.3x10^6$ to $5.3x10^8$ and LB75 ($4.8x10^6$ to $2.2x10^9$) respectively.



Parameters: DM=Dry matter; Cp=Crude protein;NDF= Neutral detergent fibre; ADF= Acid detergent fibre & MC=Moisture content

Fig 1: Comparative Composition of the Fermented Fodder

Figure 1 shows the comparative composition of the fermented fodder (Silage). After the period of fermentation, the Dry matter increased from 18.42 to 26.12, so also the crude protein (5.67 to 7.32), NPF (59.25 to 62.12), ADF (57.43 to 61.54) respectively. The moisture content and the pH decreased favourably from 40% to 35% and from 4.7 to 3.6 respectively.

Discussions

Research has shown that the fermented LAB obtained from some grasses could be used as an additive to ensile tropical grasses Bureenok *et al*., 2011). In this study, homofermentative and heterofermentative lactic acid bacteria were isolated from different fermented grasses; Elephant grass, Guinea grass and Gamba grass obtained at Giri, FCT, Abuja, Nigeria. From all the initial numbers of isolates, three of them produced low pH and were selected for controlled fermentation of pre-treated elephant grass for silage production. LAB are known to lactic acid from the fermentation of hexoses, as well as other products (acetic acid, propionic acid, or ethanol), and CO_2 that add to the preservative and nutritive qualities of animal feeds. (Chen, *et al*., 2017).

Based on both the biochemical, PCR (16S rRNA) analysis and acid production potentials, *L. plantarum* (LB09), *L. casei* (LB37) and *L. lactis* (LB75) were selected for silage making. Meanwhile, *L. plantarum* has been isolated from many kinds of grass such as king grass, vetch, tall fescue, and perennial ryegrass (Wang *et al*., 2017; Shah *et al*., 2018).

In this study, we screened thermotolerant LAB for developing a silage inoculant to be applied under tropical conditions. All LAB strains used for this study were able to grow at 45°C. Guo *et al*. (2020). It has also been documented that *L. plantarum* strain isolated from the feces of dairy cows was able to grow at 50°C. However, the maximum temperature for optimum LAB growth and reproduction should not exceed 45°C (McDonald *et al*., 1991).

Most fermentation produce heat which tend to destroy both pathogenic and silage spoilage microorganisms. Therefore, the use of thermotolerant LAB strains in the study is a potentially of interest to serve as an inoculant to achieve well-preserved silages in (sub) tropical regions. Antimicrobial compounds produced by LAB were classified as

organic acids, hydrogen peroxide, and bacteriocin-like compound (Heredia-Castro, *et a*l., 2015).

Reports by researchers such as Li *et al*. (2015) shows that some LAB strains isolated from corn stover silage had inhibitory effect against *Salmonella enterica* ATCC 43971^T, *E. coli* ATCC 11775^T, and *Micrococcus luteus* ATCC4698^T.

Silage storage determinant factors such as Dry matter content, crude protein and moisture content have variously been studied using lactic acid (Zhang *et al*., 2016). However, the natural initial LAB numbers in tropical forages are commonly too low for successful ensiling (Auerbach and Theobald, 2020).

The increase in dry matter content was due to the activities of the bacteria on the components of the grass material. In the same vein, Crude protein content increased during the fermentation process because the plant and microbial proteolytic processes in the ensiled material changes the nitrogenous compounds in silages and result in an increase in soluble N and NH_3–N (Kung *et al*., 2018).

The low pH values in all LAB-treated material were due to breakdown of component sugars into different organic acids. This have some protective value on the ensiled product in being able to inhibited the growth of clostridia, which most likely prevented excessive CP loss (Tian *et al*., 2014).

It is generally accepted that well-preserved silages should contain pH values less than 4.5 (Kung *et al*., 2018). This was achieved in this research.

Conclusions

LAB are the most commonly used microorganisms for the fermentation and preservation of foods. Their importance is associated mainly with their safe metabolic activity while growing in foods utilising available sugar for the production of organic acids and other metabolites.

The current LAB strains have the potential to inhibit the proliferation of undesirable and detrimental microorganisms, which also warrants the use of LAB in silage making.

The most common measurement of silage fermentation is pH, and when combined with dry matter, pH can adequately indicate the overall effectiveness of fermentation. If silage has undergone proper

fermentation, the expected pH will range from 3.5 to 4.5. This was also observed in this work. The results from this work show promising solutions in the Post-Covid-19 economic era. Selected LABs can be used for the production of livestock feeds in order to solve many nomadic-associated problems in this economic recovery plans, where feed costs, farmers-nomadic clash, low production and management practice needed to be reviewed in line with the available scientific skills to enable maximum economic returns.

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