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Responses of *Lactiplantibacillus plantarum* and *Candida glabrata* Isolated from Retted Cassava to Acid Stress and Their Influence on Substrate Fermentation for *Gari* Production

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Authors' contributions

This work was carried out in collaboration among all authors. Author STO designed the experiment, prepared and proofread the manuscript. Author OAA conducted the experiment, prepared and proofread the manuscript. Author COA managed the literature searches, prepared and proofread the manuscript. All authors read and approved the final manuscript.

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ABSTRACT

Aim: Fermentation subject microbial cells to stress, such as, acid stress which can lead to inactivation of cells and consequently death. Microbial cells have developed mechanisms of adapting to these stress conditions. In this study therefore, the physiological and proteomic

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responses of *Lactiplantibacillus plantarum*LC03 and *Candida glabrata*YC02 to acid stress and their influence on substrate fermentation for *Gari* production has been investigated.
Methodology: Using the turbidimetry method and SDS-PAGE; LC-MS/MS, the physiological and proteomic responses of the LAB and yeast to acid stress were assessed. Analysis of the physiocochemical and organoleptic properties of the fermented cassava using the LAB and yeast alone and in combination was conducted by means of standard methods.
Results: *Lactiplantibacillus plantarum*LC03 and *Candida glabrata*YC02 had growth at pH 1, 2 and pH 3 respectively with an increased protein intensity of Type I glyceraldehyde-3-phosphate dehydrogenase and enolase 2 respectively. The lowest cyanide content (6.49^{d}), highest protein content (0.94^{c}) and improved organoleptic acceptability (7.92^{a}) was observed in *Gari* produced with the combination of *Lactiplantibacillus plantarum*LC03 and *Candida glabrata*YC02 as starters with significant differences in *Gari* produced with single starters of *Lactiplantibacillus plantarum*LC03 and *Candida glabrata*YC02 and control.
Conclusion: Increased protein intensity during acid stress conditions enhanced the survival of *Lactiplantibacillus plantarum*LC03 and *Candida glabrata*YC02 (starters), thereby, improving the quality (improved sensory properties, nutritional and reduced anti-nutrient contents) of *Gari* produced.

Keywords: Lactiplantibacillus plantarum; Candida glabrata; retted cassava; starters; acid stress.

1. INTRODUCTION

Cassava fermentation (retting) for the production of '*gari'* involves the spontaneous fermentation of grated or sliced pieces of cassava at room temperature for 4 to 6 days [1]. Lactic acid bacteria (species of *Lactobacillus, Lactococcus, Streptococcus, Corynebacterium* and *Leuconostoc*) [2,3] and yeast (*Candida and Cryptococcus* species) are involved in fermentation of cassava for the production of *gari* resulting in the lowering of the pH of the fermenting medium [3]. *Lactiplantibacillus plantarum* have been reported as the most predominant lactic acid bacteria (LAB) in fermenting cassava [4]. Work done by Edward et al. [3] showed that *Candida* species were common occurrence in fermenting cassava for the production of *gari*. During fermentation, organic acids are produced by most microbes as either products or end-products [5]. Organic acids such as propanoic, lactic, butanoic and acetic acid amongst other acids are produced by fermenting LAB and yeasts in the course of fermentation, which are thought to bring about the reduction in pH and a distinctive aroma of fermented cassava foods [6].

The organic acid accumulated further acidifies the environment for microbial growth, thus having a negative impact on microbial productivity and volume of bioprocesses, with increasing concentration of the acids [7,5]. Increased intracellular acidity, as well as an accelerated metabolic disorder of the cells occurs as protonated acids enter the cell, dissociating into proton and corresponding ion [8, 9]. The reaction of microbes to vital changes in environmental conditions, natural stress and stress conditions experienced during food processing is known as microbial stress response [10]. Microbial stress responses is described by the physiological changes and the transitory stimulation of general and specific proteins that are largely responsible for the organism's ability to survive the harsh conditions of the environment. Different metabolic pathways (anabolic and catabolic pathways) are commonly activated as an adaptation (protective) mechanism to the stress condition (such as acid, heat, salt, osmotic, starvation stress) confronting the organism, which results in changes in the organism's protein profile [11].

The injury triggered by acidic environments is alleviated through cell membrane integrity and fluidity, pH homeostasis maintenance, macromolecule repair and regulation of metabolism. The methods of acid tolerance can be exploited in the protection of probiotics against acids in the course of food ingestion, as well as the development of biosynthesis of organic acids [12]. The method of acid stress response which relies on synthesis of protein has been reported in microbes [13]. Certain proteins are typically induced as a result of acid stress in order to protect or repair DNA and proteins. A number of chaperones are well-known and are essential for production, transfer, fold-up and breakdown of proteins [14]. Microbes are capable of changing their metabolic and energy fluxes, adjust growth rate and synthesis of

enzymes and metabolites, so as to modify the carbon metabolism to the new environment [15].

Small membrane heat shock protein was characterized in *Oenococcus oeni* which improved acid tolerance by suppressing the clumping of protein [16]. Under acid stress conditions, *Lactobacillus casei* and *Streptococcus mutans* significantly decreased the production of phosphoenolpyruvate phosphotransferase system (PEP-PTS) for glucose [17, 18]. Similarly, at low pH, an increase in glucose phosphotransferase system was reported in *Streptococcous sobrinus* [19] and *Streptococcus macedonicus* [20]. An increased abundance of bifunctional acetaldehyde-CoA/alcohol dehydrogenase, 30S ribosomal protein S2 and 50S ribosomal protein L5 was observed in *Lactobacillus amylovorous* at low pH. Also, *Candida kefyr* showed an increased abundance of 6-phosphogluconate dehydrogenase at low acid conditions [21]. High levels of these proteins may confer acid resistance.

In the course of fermentation of retted cassava to produce *Gari*, LAB and yeasts exposed to stress conditions which can bring about reduction in the viability and reproduction of the fermenting microbes, as well as the organoleptic and fermentative qualities. Responses of LAB and yeast to stress during the production of *Gari* is important in order to understand the physiological and proteomic response of the fermenting microbes to the stress conditions, as well as improve the utilization of these microbes for fermentation. This research studies the responses of *Lactiplantibacillus plantarum* and *Candida glabrata* to acid stress (low pH) and their influence on substrate fermentation (nutritional, anti-nutritional and organoleptic properties) for *Gari* production.

2. MATERIALS AND METHODS

2.1 Sample Collection

Fresh cassava samples were collected from Water Area, University of Ibadan Road, Ibadan, Nigeria and were transferred to the laboratory for the laboratory preparation of '*Gari*' [22].

2.2 Isolation of Lactic Acid Bacteria and Yeast

In this study, *Lactiplantibacillus plantarum* and *Candida glabrata* which had formerly been isolated and characterized using morphological, physiological and biochemical tests, Matrix Assisted Laser Desorption Ionization Time of Flight Mass Spectrometry, (MALDI TOF MS) by means of the formic acid extraction method were used [23].

2.3 Maintenance of Lactic Acid Bacteria and Yeast

The maintenance of *L. plantarum* and *C. glabrata* was done according to the method of [24]. Pure culture of *L. plantarum* was grown in new MRS (De Mann Rogosa Sharpe) agar slants micro-aerobically at 30^{o}C for 3 days. Also pure culture of *C. glabrata* was grown in new YPD (Yeast Extract Peptone Dextrose) agar slants at 25^{o}C for 2 days. After visible growth, the LAB and yeast were preserved at 4^{o}C for routine use, but sub-cultured every 4 weeks.

2.4 Responses of *L. plantarum* and *C. glabrata to* Acid Stress at Different pH Concentrations

2.4.1 Physiological response

"Five (5) µL of fresh broth culture *L. plantarum* and *C. glabrata* were introduced into 250 µL of MRS and YPD broth respectively (Sigma Aldrich) adjusted to pH 1, 2, 3 and 4 in micro-plates. The micro-plates inoculated with *L. plantarum* were grown micro-aerobically at 30^{o}C for 24 h while the micro-plates inoculated with the *C. glabrata* were grown at 30^{o}C for 24 h. The experimental setup were repeated three times. With the use of microplate reader (Beck Coulter) at 620 nm, the growth of cultures were read and recorded at the end of 24 h" [25].

2.4.2 Proteomic response

2.4.2.1 Protein extraction from L. plantarum and C. glabrata subjected to acid stress

Lactiplantibacillus plantarum was grown in MRS broth with acid stress conditions (MRS broth adjusted to pH 1, 2, 3, 4) and MRS broth without acid stress conditions (control) to examine the acid stress response and were then cultured at 30^{o}C for 24 h. Also, fresh culture of *C. glabrata* were grown in YPD broth subjected to acid stress at pH 1, 2, 3, 4 and YPD broth without acid stress conditions (control) and cultured at 25^{o}C for 24 h. "At the end of incubation, proteins extraction from *L. plantarum* and *C. glabrata* was carried out. 100 ml of *L. plantarum* and *C. glabrata* cells at the different stress conditions and control were taken and centrifuged at 4200

rpm (Beckman Coulter, Allegra X-22 Centrifuge) for 20 min, while the resulting liquid was thrown out. Collected pellets were washed thrice in phosphate buffer saline (PBS) solution (10 ml), centrifuged at 4200 rpm for 10 min each. The weight of individual cell pellet in the Eppendorf tubes was obtained and estimated to give the volume of thiourea lysis buffer (4% Chaps, 2 M Thiourea, 1 % DTT, 7M Urea, 2% carrier ampholytes pH 3-10, 1 M Tris base, 10mg Protease inhibitor) to be added to each cell pellet. Lysing of cells were carried out using acid washed glass beads of about 212 to 300 µm diameter with thorough vortexing on table mixer (Maxi Mix II, Barnstead Thermolyne, USA) at a maximum speed approximately 2500 rpm with intermittent placement of the cell pellets on ice cubes for 1 min interval. The cells previously lysed were subsequently re-solubilized by ultra-sonication (Ultrasonic bath- Bio-equip 393 × 407) on ice for 6 rounds at 15 sec each. Samples were cooled on ice for a min in between sonication to completely solubilize precipitated proteins. Removal of cell remains was done by centrifuging the cells at 4800 rpm for 10 min at 4^{o}C. The resultant supernatants which contain the protein fraction was quantified and was then used for sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE)" [26].

2.4.2.2 SDS PAGE and protein detection

"Protein samples extracted from *L. plantarum* and *C. glabrata* previously subjected to acid stress conditions was used to conduct the SDS PAGE using 12% acrylamide resolving gel and stacking gels" [27]. "Staining and destaining of gels was carried out in order to detect the protein by using Coomassie Brilliant Blue R-250" [28].

2.4.2.3 Peptide extraction, MS analysis and protein identification

Bands which showed higher or lower band intensity were excised from the gels for protein extraction [29], peptides were analyzed using the Liquid Chromatography Mass Spectrometry (LC MS/MS) [30] and identification of protein was done [31].

2.5 Preparation of Laboratory Produced Gari Using *L. plantarum* and *C. glabrata* as Starter Cultures, Fermentation and Data Analysis

Laboratory prepared '*Gari* using grated cassava was carried out and inoculated with pure cultures of *L. plantarum*, *C. glabrata* in singly and the combination of *L. plantarum* and *C. glabrata* [32]. The grated cassava '*Gari'* inoculated with *L. plantarum*, *C. glabrata* and the combination of *L. plantarum* and *C. glabrata* were allow to undergo fermentation at 28± 2^{o}C for a duration of 72 h. At the start and end of fermentation, the pH of inoculated grated *cassava* samples were recorded employing a Jenway pH meter; the proximate, mineral and anti-nutrient analysis of the samples were evaluated. The proximate composition (moisture content, neutral detergent fibre, acid detergent fibre, ash content, fat extract content, nitrogen free extract, crude fibre) and mineral content (potassium, manganese, iron, sodium, calcium, magnesium, zinc, phosphorous and copper) were evaluated [33],. The tannin content, phytate and alkaloids content of the fermented samples were analyzed [34] and the cyanide content of fermented samples were analyzed [35]. "Also, the organoleptic characteristics of the samples were examined to investigate the acceptability of the product. With the use of a panel group consisting of twenty members conversant with drinking *Ogi*, the organoleptic properties (including appearance, texture, flavour, and general acceptability) of fermented samples was accessed with the use of the 9- point hedonic scale method varying from 9 signifying "like exceptionally" to 1 indicating "dislike exceptionally. The laboratory produced *Ogi* was assessed by the panel members, thus indicating out the level of preference for each sample" [36]. Results gotten from the pH determination, proximate, mineral, anti-nutrient content and organoleptic properties of laboratory produced *Ogi* using the starter cultures were analysed statistically using ANOVA and means were separated using Duncan multiple range test at α 0.05.

3. RESULTS AND DISCUSSION

Fig. 1 shows the physiological response of *L. plantarum* isolated from fermented cassava subjected to acid stress. "An increase in growth of *L. plantarum* was observed as the pH of the growth medium increases from pH 1, 2, 3, to 4. The highest growth was recorded at pH 4 (0.659) while the lowest growth was observed at pH 1 (0.257). The physiological response of *L. plantarum* to acid stress showed that the growth of the LAB increased as the pH of the medium increased. The research on the response of *L. amylovorus* to different pH concentrations revealed an increase in the growth of *L. amylovorus* as the pH of the medium increased"

[21]. Research work on bile and acid tolerance of *L. plantarum* KCA-1 showed that *L. plantarum* KCA-1 was tolerant at pH 2.5 and a corresponding increase in the growth of *L. plantarum* KCA-1 was recorded at pH 4.5 [37].

The physiological response of *C. glabrata* isolated from fermented cassava subjected to acid stress at different acid concentrations is shown in Fig. 2. *Candida glabrata* grew at pH 1, 2, 3, and 4 and recorded an increase in growth as the pH of the medium increased. At pH 4, *Candida glabrata* recorded the highest growth (1.281) while at pH 1, the lowest growth (0.399) of the yeast was recorded. From this study, the physiological response of *Candida glabrata* to acid stress at pH 1, 2, 3 and 4 revealed an increase in the growth of the yeast as the pH increases, with an optimum growth at pH 4 and thereafter a decline in the growth concentration from pH 4 to 1. The effect of low pH on the growth of *Candida kefyr* isolated from *Ogi*, fermented sorghum gruel was studied and it was noted that the growth of *Candida kefyr* gradually decreased as the pH of the medium was lowered [21]. Similar observations reported the growth of *Candida glabrata* MTCC 3987 at low acidic pH [38].

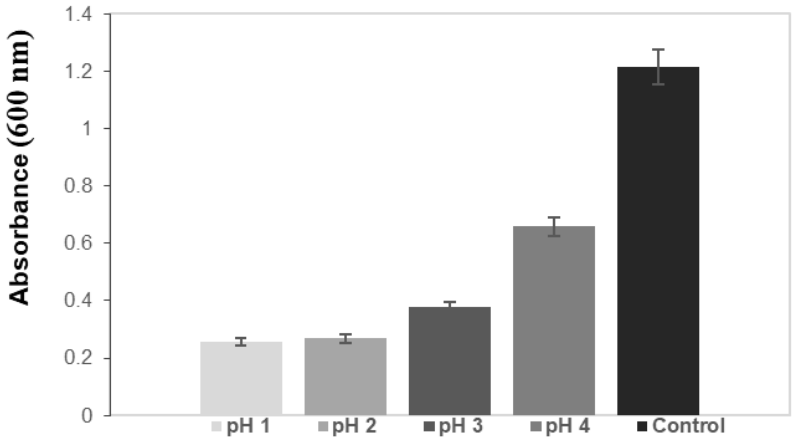

Fig. 1. Growth of *Lactiplantibacillus plantarum* subjected to acid stress at different pH concentrations

X – axis label refers to the pH concentrations at pH 1, 2, 3, 4.
The absorbance at 600 nm (+ SD) after 24 h of growth of L. plantarum subjected to acid stress at different pH concentration refers to Y – axis.
No pH adjustments were included in the control media

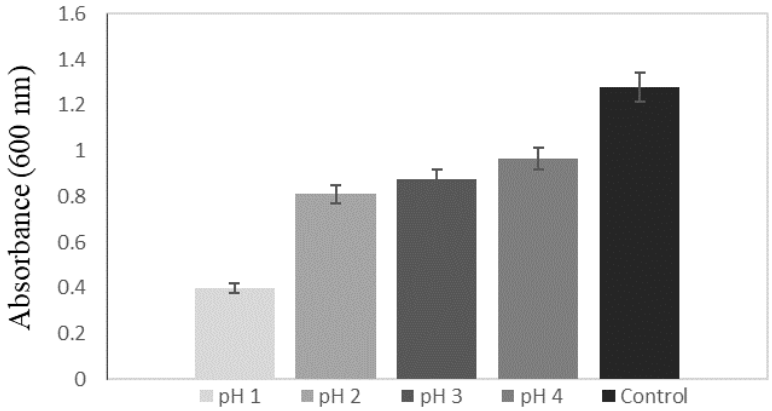

Fig. 2. Growth of *Candida glabrata* subjected to acid stress at different pH concentrations

X – axis label refers to the pH concentrations at pH 1, 2, 3, 4.
The absorbance at 600 nm (+ SD) after 24 h of growth of C. glabrata subjected to acid stress at different pH concentration refers to Y – axis. No pH adjustments were included in the control media

"Plate 1 represents the SDS PAGE of *L. plantarum* isolated from fermented cassava subjected to acid stress at different pH concentrations. Three gel bands, F6, G7 and H8 were cut, analyzed and identified as Type I glyceraldehyde-3-phosphate dehydrogenase, chitin-binding protein and elongation factor Tu respectively. It was observed that protein gel band F6 which recorded an approximate molecular weight of 43.3 kDa, showed more band intensity at pH 1 and 2 compared to acid stress conditions at pH 3 and 4 and control (Lane C). Also, protein gel band G7 obtained from the SDS PAGE of *L. plantarum* which had an approximate molecular weight, 36.6 kDa showed more intensity at pH 2 and 3 than at pH 1, 4 and control. More protein gel band intensity was observed in H8 (approximate molecular weight, 22.3 kDa) at pH 2 compared to pH 1 and 3 while low protein gel band intensity was noted at pH 4 and Control. Responses to acid stress in *L. plantarum* isolated from fermented cassava revealed a notable expression and repression of Type I glyceraldehyde-3-phosphate dehydrogenase, chitin-binding protein and elongation factor Tu. Expression of these proteins indicates the response of *L. plantarum* to stress. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is important in glycolysis as it results in the oxidative production of 1, 3-diphosphoglycerate from phosphorylation of glyceraldehye 3-phosphate. GAPDH also function in apoptosis, duplication and management of DNA, endocytosis and control of messengerRNA" [39]. "The relationship of GAPDH to the cell envelope of Lactobacilli has been studied" [40]. "Remarkably, the connection of the membrane of *L. crispatus* at low pH, while at neutral pH of 7, GAPDH is quickly discharged into the medium which work together with lipoteichoic acid has been noted" [41]. "Inactivation or reduced activity of GAPDH reduces glycolysis and metabolism in tricarboxylic acid cycle. Hence, there is a redirection of glucose to the pentose phosphate pathway, thereby producing additional NADPH, which is required by antioxidant enzymes" [42].

The other differentially expressed protein in *L. plantarum* is elongation factor thermo unstable (EF-Tu). The low intensity of EF-Tu during acid stress is noted in this study. Previously, notable decrease in the intensity of 30 S ribosomal protein S2 and 50 S ribosomal L5 during acid stress in *L. amylovorus* isolated from fermented sorghum gruel was reported [21]. In several LAB, EF-Tu was localized to the cell wall and known as a moonlighting protein which is regarded as a protein with several unconnected roles at diverse location of the cell [43]. The function of EF-Tu in the synthesis of protein is involved in the transportation of aminoacyl-transportRNA complex and facilitates its binding to the ribosome (A site) [44]. Secondly, it protects other proteins from aggregating by attaching to the water-repelling areas of the altered protein; this shows the chaperone activity of elongation factor Tu [45]. Thirdly, isomerase activity (protein disulfide) is demonstrated by elongation factor Tu. When compared to other protein disulfide oxidoreductases, EF-Tu activates the establishment of disulfide bond, protein reduction and isomerization [46]. EF-Tu is also involved in protein folding as well as defense in microbial cell during stress in addition to its vital functions in translation and protein elongation [47]. The excessive expression of EF-Tu in the course of adaptation to acid stress has been described in *Propionibacterium freudenreichii* and *Streptococcus mutans* [48], also in adaptation of *Listeria monocytogenes* to osmotic stress [49].

Furthermore, from this study, an increased intensity of chitin-binding protein (CBP) has been noted during acid stress at pH 1, 2 and 3 in *Lactiplantibacillus plantarum*. Previously, three *L. plantarum* strains produced some proteins extracellularly within the medium with unascertained roles, of which a few of the proteins were correspondingly found in the guts [50]. Additionally, more investigators have in recent times spent colossal attempts to discover surface-related proteins with a probable function as regards coherence of *Lactiplantibacillus plantarum* and its environs [31]. Chitinization process in *L. lactis* has been reported to be made up of chitinases and chitin binding proteins showing similarity to those from *Lactiplantibacillus plantarum* [51]. Chitin binding proteins are termed as complement proteins that do not produce hydrolysis and are important for the breakdown of chitin which attach to N-acetylglucosamine existing in varied polymers, in addition to chitin, also mucins [52,53].

The SDS PAGE of *C. glabrata* isolated from fermented cassava subjected to acid stress at different pH concentrations is represented in plate 2. Protein gel bands I9 and J10 were cut, analyzed and identified. It was noted that the protein gel band I9 which recorded an approximate molecular weight of 46.3 kDa showed increased protein band intensity at pH 2, 3 and 4 compared to the control (no acid stress conditions included). Also, protein gel band J10

with an approximate molecular weight of 27.5 kDa showed increase in the protein band intensity at pH 2 compared to acid stress conditions at pH 3 and 4. However, no increase or decrease in protein band intensity was noted in protein gel band I9 and J10 at pH 1. The results obtained from the protein analysis of *Candida glabrata* isolated from fermented cassava in this study showed the identification of two proteins; enolase 2 and an uncharacterized protein. From this study, an increase in the abundance in enolase 2 was observed during acid stress. This possibly will bring about a more rapid level in glycolysis and tricarboxylic acid cycle as a glycolytic enzyme during encounter with acid stress conditions. Likewise, uncharacterized protein showed a lower level of abundance during acid stress compared to the glycolytic enzyme; enolase 2. Previous work reported an increase in the abundance of 6-phosphogluconate dehydrogenase and enolase in *Candida kefyr* isolated from fermented sorghum gruel during acid stress [21].

Table 1 shows the identification of protein using the LC ESI MS/ MS analysis from protein gel bands of *Lactiplantibacillus plantarum* and *Candida glabrata* isolated from fermented cassava. Proteins from gel bands F6, G7 and H8 obtained from *L. plantarum* recorded the number of peptide matches as 652, 542 and 99 respectively and a corresponding emPAI of 77.07, 23.48 and 33.27 respectively with a score range of 4992 and 41098. The proteins obtained from gel bands D4, E5 and F6 were identified as type 1 glyceraldehyde 3 phosphate dehydrogenase, elongation factor Tu and chitin-binding protein respectively with a reference organism from *L. plantarum*. Meanwhile, the proteins from gel band I9 and J10 obtained from *C. glabrata* were identified as enolase 2, uncharacterized protein, with reference organism from *C. glabrata*. The number of peptide matches obtained from I9 and J10 using the LC-ESI-MS/ MS analysis were 834 and 393 and an emPAI of 76.77 and 81.10 respectively.

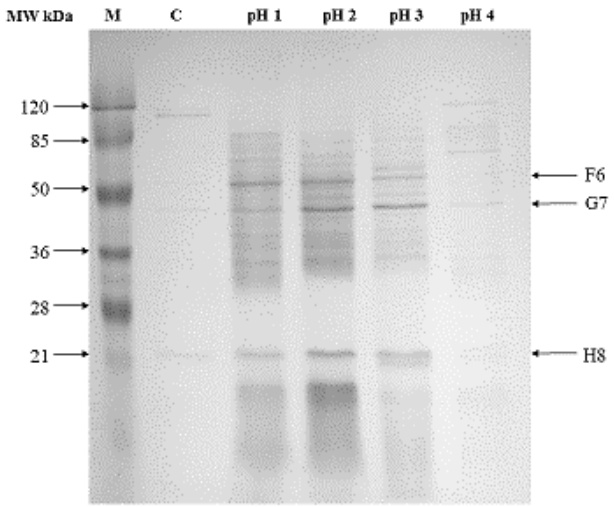

Plate 1. SDS PAGE of *Lactiplantibacillus plantarum* isolated from fermented cassava subjected to acid stress at different pH concentrations

Key

**MW: Molecular weight *kDa: kilo Daltons *M: Molecular marker*

**C: Control *F6, G7, H8: Excised and identified bands.*

X – axis label refers to pH concentrations at pH 1, 2, 3, 4 at which proteins were extracted from Lactiplantibacillus plantarum after 24 h in growth medium. Y- axis label refers to molecular weight (MW) (kDa) of the molecular marker; which acts as the marker for the approximate molecular weight of protein gels. No pH adjustments were included in the control medium. With the use of LC ESI MS/MS analysis, protein gels F6, G7 and H8 were excised and identified

Table 1. Identification of protein characterized by LC ESI MS/MS analysis from *Lactiplantibacillus plantarum* and *Candida glabrata* isolated from fermented cassava to acid stress

Band/spot code	Organism origin	Database	Accession number	Mass (kDa)	PI	No of peptide matches	No of sequences	emPAI	Score	Protein identity	Organism/Reference
Band-F6	*Lactiplantibacillus plantarum*	NCBIprot	WP_024521321.1	36.641	-	652	36	77.07	41098	Type I glyceraldehyde-3-phosphate dehydrogenase	*Lactiplantibacillus plantarum*
Band-G7	*Lactiplantibacillus plantarum*	NCBIprot	WP_044431076.1	43.364	-	542	25	23.48	29526	Elongation factor Tu	*Lactiplantibacillus plantarum*
Band-H8	*Lactiplantibacillus plantarum*	NCBIprot	WP_076633771.1	22.368	-	99	15	33.27	4992	Chitin-binding protein	*Lactiplantibacillus plantarum*
Band-I9	*Candida glabrata*	NCBIprot	SCV15549.1	46.362	-	834	42	76.77	27747	Enolase 2	*Candida glabrata*
Band-J10	*Candida glabrata*	NCBIprot	XP_445966.1	27.574	-	393	28	81.10	22619	Uncharacterized protein	*Candida glabrata*

Key:
NCBI- National center for biotechnology information
NCBIprot- database for protein, a pool of sequences from some resources comprising translation from noted coding segment in GenBank and TPA, along with SwissProt, PIR, PRF and PDB
Accession number: chains of figures that are allocated successively to every single sequence data sorted by NCBI.
Mass (kDa): theoretic mass expected from the amino acid series of the known protein.
pI: theoretic isoelectric point expected from the amino acid series of the known protein
Number of sequence: analysis of the amino acid series of the known protein
Number of peptide matches: total of corresponding peptides centered on MS/MS data searching, without the replica corresponds.
emPAI is the Exponentially Modified Protein Abundance Index
Organism reference: organism discovered after alignment of the known protein series.

Table 2. Changes in pH of laboratory prepared *Gari* using starter cultures

pH	Ctrl	Ogi produced with Starter cultures			P Value	SEM
		C. gla	*L. pla*	*C. gla + L. pla*		
0 h	6.74^{a}	6.67^{b}	6.41^{d}	6.59^{c}	0.001	0.03
72 h	4.46^{a}	4.01^{b}	3.81^{d}	3.96^{c}	0.001	0.09

Values are the means of triplicate determinations. Means along the rows with distinct superscript are substantially distinct from each other at $\alpha_{0.05}$.

Key:
**Ctrl-Control (spontaneous fermentation) *C. gla - Candida glabrata *L. pla - Lactiplantibacillus plantarum *C. gla + L. pla - Candida glabrata + Lactiplantibacillus plantarum *P value- Probability value *SEM-Standard Error Mean*

Table 2 represents the pH changes in laboratory prepared Gari using *Lactiplantibacillus plantarum* and *Candida glabrata* and the combination of *Lactiplantibacillus plantarum* and *Candida glabrata* as starter cultures in the course of fermenting cassava. Decreased pH were recorded in fermented cassava prepared using the starter cultures and the control (spontaneous fermentation). The changes observed in the pH of fermented cassava made using the single starter culture *L. plantarum* recorded the lowest pH (3.81^{d}) compared to fermented cassava produced using the single starter culture *C. glabrata* (4.01^{b}) and the combination of *L. plantarum* and *C. glabrata* (3.96^{c}) and the control (spontaneous fermentation) (4.46^{a}) (Table 2). Previous works have reported the use of lactic acid bacteria and yeasts as starter cultures in the production of fermented foods [54, 55, 56]. Fermentation of grated cassava using *L. plantarum* and *C. glabrata* as starter cultures was characterized by a fall in pH which was noticed during the course of fermentation. The lowering of pH through application of starters has been described in some fermented cereal beverages [57, 58, 55]. Reduction in pH arose from rising hydrogen ion content, perhaps owing to the activities of microorganisms in breaking down sugar and more existing food items to yield organic acids [59].

The proximate composition of laboratory prepared *Gari* using starter cultures is represented in Table 3. The results obtained from the proximate composition of laboratory prepared *Gari* showed substantial variations as regards the protein content, ash content ADF and NDF *Gari* produced using the combined starters of *Lactiplantibacillus plantarum* and *Candida glabrata* compared to *Gari* produced with single starter cultures of *Lactiplantibacillus plantarum* and *Candida glabrata.* The highest value of protein content (0.94^{c} %) and ash content (2.80^{a} %) was observed in fermented cassava made with combined starters of *Lactiplantibacillus plantarum* and *Candida glabrata* compared to the single starters of *Candida glabrata* (0.85^{b} % and 2.56^{c} % respectively) and *Lactiplantibacillus plantarum* (0.90^{a} % and 2.64^{b} %). *Gari* produced with single starter *Candida glabrata* recorded the highest moisture content (8.55^{a} %), while *Gari* produced with combined use of the starter cultures *Lactiplantibacillus plantarum* and *Candida glabrata* recorded the lowest moisture content (6.08^{b} %). It was observed that no significant variation was noted in the fat content and NFE of *Gari* produced with single starter *Lactiplantibacillus plantarum* and the combined use of *Lactiplantibacillus plantarum* and *Candida glabrata* (Table 3). From this work, notable increase in protein content of *Gari* produced using combined starters of *Lactiplantibacillus plantarum and Candida glabrata* during fermentation of grated cassava compared to using single starters and the control (spontaneous fermentation) has been reported previously. Reports showed an improvement in the protein content of *Ogi* (a fermented sorghum gruel) produced using combined starters of *Lactobacillus amylovorus and Candida kefyr* [21] Also, a three-fold increase in the crude protein content was recorded in fermented cassava after a fermentation period of 48 h [60]. Similar work reported an improvement in the crude protein content (from 2% to 34.5 %) of cassava chip fermented with *Saccharomyces cerevisiae* as single starter culture [61]. The secretion of extracellular enzymes and the action of microbial growth may account for the increase in protein content during fermentation using starter cultures [62]. In the same vein, increased ash content observed in *Gari* produced using combined starters of *Lactiplantibacillus plantarum and Candida glabrata* during fermentation of grated cassava compared to using single starters and the control (spontaneous fermentation) may perhaps be as a result of the activities of enzymes and microbes during fermentation resulting in the dissipation of dry matter [63].

Similarly, increased fat content was observed in *Ogi* produced with the combined starters of *Lactobacillus amylovorus and Candida kefyr* during fermentation of sorghum gruel compared to the *Ogi* produced using single starters and control [21].

Also, reduced fat content noted in *Gari* produced using the combined starter cultures of *Lactiplantibacillus plantarum and Candida glabrata* compared to the use of single starters and the control (spontaneous fermentation) is in line with work done by [64, 65, 21]. The action of enzymes (lipolytic) during the process of fermentation could be accredited to the notable decrease in the fat content.

Furthermore, improved crude fibre content (neutral detergent fibre and acid detergent fibre) of *Gari* produced using the single starter cultures and the combined starter cultures with significant differences was noted compared to the control. Higher crude content was reported in *Ogi* produced using combined starter cultures of *Lactobacillus amylovorus and Candida kefyr* [21]. Previous reports have stated the important benefits of consuming dietary fibre in diet [66, 67, 68, 69].

Table 4 shows the mineral composition of laboratory prepared *Gari* using starter cultures. *Gari* produced using joined starters of *Lactiplantibacillus plantarum* as well as *Candida glabrata* recorded the utmost mineral content in calcium (2.06^{a} %) and zinc (0.00075^{a} %) which showed significant differences compared to the *Gari* produced using single starter cultures *Lactiplantibacillus plantarum*; calcium (0.08^{b} %) and zinc (0.0004^{b} %) and *Candida glabrata*; calcium (0.09^{b} %) and zinc (0.00035^{b} %) following the results of the analysis of variance. The highest potassium content was observed in *Gari* produced using single starter culture of *Lactiplantibacillus plantarum* (1.05^{a} %) which showed no significant difference compared to *Gari* made with single starter of *Candida glabrata* (0.97^{b} %) and joined starters of *Lactiplantibacillus plantarum* as well as *Candida glabrata* (0.98^{b} %) and control (0.95^{b} %) (Table 4). Increased mineral composition of *Gari* produced with the starter cultures were noted in the study. Results obtained from this research disclosed that the produced *Gari* with the use of *Lactiplantibacillus plantarum* and *Candida glabrata* recorded better and more improved mineral composition compared with *Gari* made with single starter cultures. Precious research reported improved mineral content with the use of starter cultures in the production of *Burukutu* [55] and Ogi [21].

The anti-nutrient composition of laboratory prepared *Gari* using starters is represented in Table 5. In correspondence to the outcome of the analysis of variance, the anti-nutrient composition of *Gari* produced without starter culture (control) recorded the highest anti-nutrient components which showed significant differences to anti-nutrients composition of *Gari* produced with starter cultures. The lowest tannin (0.0007^{d} %), phytate (0.0078^{c} %), alkaloids (0.14^{c} %) and cyanide (6.49^{d} mg/kg) was observed in *Gari* made using joined starters of *Lactiplantibacillus plantarum* and *Candida glabrata* compared to *Gari* made using single starters of *Lactiplantibacillus plantarum* or *Candida glabrata* (Table 5). A notable decrease in the anti-nutrient components including phytate, tannin, total alkaloids and cyanide was observed in *Gari* produced using both the single starter cultures and the combined starter cultures. Anti-nutrients are capable of interfering with the assimilation of important food nutrients and minerals during digestion [70], although, some studies have reported the positive attributes of some anti-nutrients [71, 72, 73]. Reduced anti-nutrient composition of tannins, polyphenols and phytate was reported in *Burukutu* (a Nigerian fermented alcoholic beverage) produced using combined starter cultures of *L. fermentum* I and *S. cerevisiae* [54]. Other research findings have reported that *Ogi* produced using single and combined starter cultures of *Lactobacillus amylovorus* and *Candida kefyr* recorded reduced anti-nutrient content compared with *Ogi* produced without the use of starter cultures [21]. Reductions in phytate and tannin content during and at the end of fermentation of cassava for the production of Ikivunde and Inyanga have also been investigated [74].

Also, reports have shown that fermentation of cassava may result in the reduction of cyanide [60, 75]. The reduction of cyanide observed in *Gari* produced using both the single starters and the combined starter cultures in this work is in line with the report of [76, 60, 75]. The notable reduced cyanide content may be as a result of the ability of the starter cultures to secrete the enzyme linamarase which subsequently hydrolyze linamarin, resulting in the degradation of cyanogenic glycosides to HCN which is further broken down to formamide and is utilized as source of carbon and nitrogen [76, 75].

Table 6 shows the organoleptic properties of laboratory prepared *Gari* produced using starters. The outcome of the variance analysis disclosed remarkable changes of mean scores of preference (flavor, texture, appearance and general overall acceptability) in *Gari* made using joined starters of *Lactiplantibacillus plantarum* and *Candida glabrata*. The least mean score of preference (flavor 6.94^{c}, texture 7.63^{c}, appearance 6.29^{d}, general overall acceptability 7.13^{d}) was observed in *Gari* produced with no starter culture (control) compared to *Gari* produced with combined and single starter cultures. In this study, the organoleptic attributes of *Gari* produced using the combined starter cultures of *Lactiplantibacillus plantarum* and *Candida glabrata* showed an improvement in the texture, flavor, appearance and general acceptability of the product. Some research findings have proposed that the use of starters for fermentation enhances the flavor of the products [77, [78]. Also, improvement in the flavor, appearance texture and general acceptability was recorded in fermented foods produced using starter cultures [55, 21].

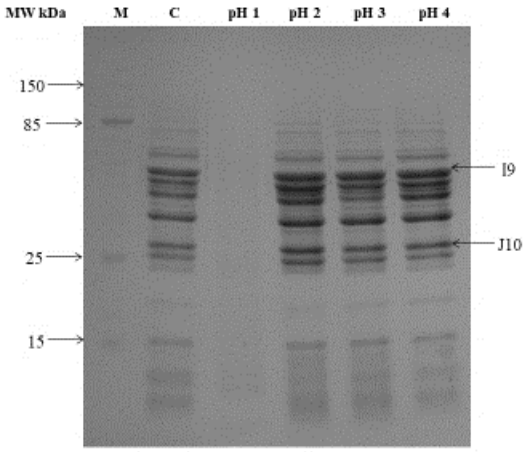

Plate 2. SDS PAGE of *Candida glabrata* isolated from fermented cassava subjected to acid stress at different pH concentrations

Key
**MW: Molecular weight *kDa: kilo Daltons *M: Molecular marker *C: Control *I9,J10: Excised and identified bands.*
X – axis label refers to pH concentrations at pH 1, 2, 3, 4 at which proteins were extracted from Candida glabrata after 24 h in growth medium. Y- axis label refers to molecular weight (MW) (kDa) of the molecular marker; which acts as the marker for the approximate molecular weight of protein gels. No pH adjustments were included in the control medium. With the use of LC ESI MS/MS analysis, protein gels I9 and J10 were excised and identified.

Table 3. Proximate composition of laboratory prepared *Gari* using starter cultures

Proximate composition (%)	Ctrl	Starter cultures			P Value	SEM
		C. gla	*L. pla*	*C. gla + L. pla*		
Moisture	6.32^{d}	8.55^{a}	6.45^{c}	6.08^{b}	0.0001	0.362
Protein	0.91^{a}	0.85^{b}	0.90^{a}	0.94^{c}	0.0001	0.019
Ash	2.31^{d}	2.56^{c}	2.64^{b}	2.80^{a}	0.0001	0.267
Fat	0.39^{c}	0.41^{c}	0.66^{a}	0.54^{b}	0.0001	0.360
NFE	0.15^{a}	0.14^{a}	0.63^{a}	0.13^{a}	0.055	0.122
ADF	5.87^{d}	6.77^{b}	7.47^{a}	7.66^{c}	0.001	0.218
NDF	7.64^{d}	11.15^{b}	12.90^{a}	13.84^{c}	0.001	0.840

Values are the means of triplicate determinations. Means along the rows with distinct superscript are substantially distinct from each other at α 0.05.

Key:
**Ctrl-Control (spontaneous fermentation) *C. gla- Candida glabrata *L. pla - Lactiplantibacillus plantarum *C. gla + L. pla - Candida glabrata + Lactiplantibacillus plantarum *P value- Probability value *SEM-Standard Error Mean *NFE - Nitrogen Free Extract *ADF – Acid Detergent Fibre *NDF - Neutral Detergent Fibre*

Table 4. Mineral composition of laboratory prepared *Gari* using starter cultures

Mineral composition (%)	Ctrl	Starter cultures			P Value	SEM
		C. gla	*L. pla*	*C. gla + L. pla*		
Calcium (Ca)	0.06[b]	0.09[b]	0.08[b]	2.06[a]	0.0001	0.170
Magnesium (Mg)	0.06[a]	0.07[a]	0.07[a]	0.07[a]	0.242	0.043
Potassium (K)	0.95[b]	0.97[b]	1.05[a]	0.98[b]	0.0001	0.110
Sodium (Na)	0.11[a]	0.11[a]	0.09[a]	0.05[b]	0.0001	0.010
Phosphorous (P)	0.05[d]	0.05[d]	0.05[d]	0.06[d]	0.381	0.001
Zinc (Zn)	0.0000[c]	0.00035[b]	0.0004[b]	0.00075[a]	0.0001	1.013
Copper (Cu)	0.0002[a]	0.0003[a]	0.00025[a]	0.00025[a]	0.381	0.189
Manganese (Mn)	0.0011[b]	0.0017[a]	0.00125[b]	0.00165[a]	0.0001	0.944
Iron (Fe)	0.0017[d]	0.00155[d]	0.00145[d]	0.00165[d]	0.173	0.441

Values are the means of triplicate determinations. Means along the rows with distinct superscript are substantially distinct from each other at α 0.05.

Key:
**Ctrl-Control (spontaneous fermentation) *C. gla- Candida glabrata *L. pla- Lactiplantibacillus plantarum *C. gla + L. pla- Candida glabrata + Lactiplantibacillus plantarum *P value- Probability value *SEM-Standard Error Mean*

Table 5. Anti-nutrient composition of laboratory prepared *Gari* using starter cultures

Anti-nutrient composition (%)	Ctrl	Starter cultures			P Value	SEM
		C. gla	*L. pla*	*C. gla + L. pla*		
Tannin	0.0018[a]	0.0014[b]	0.0011[c]	0.0007[d]	0.005	0.00015
Phytate	0.0093[a]	0.0089[b]	0.0086[b]	0.0078[c]	0.002	0.00019
Alkaloids	0.17[a]	0.15[b]	0.14[c]	0.14[c]	0.0001	0.00235
Cyanide (mg/kg)	7.11[a]	6.59[b]	6.53[c]	6.49[d]	0.0001	0.44015

Values are the means of triplicate determinations. Means along the rows with distinct superscript at each starter cultures are substantially distinct from one other at P< 0.05.

Key:
**Ctrl-Control (spontaneous fermentation) *C. gla- Candida glabrata *L. pla- Lactiplantibacillus plantarum *C. gla + L. pla- Candida glabrata + Lactiplantibacillus plantarum *P value- Probability value *SEM-Standard Error Mean*

Table 6. Organoleptic properties of laboratory produced *Gari* using starter cultures

Organoleptic properties	Ctrl	*Starter cultures*			P Value	SEM
		C. gla	*L. pla*	*C. gla + L. pla*		
Flavor	6.94[c]	8.18[b]	7.87[b]	8.82[a]	0.001	0.258
Texture	7.63[c]	7.72[ab]	7.69[bc]	7.84[a]	0.013	0.022
Appearance	6.29[d]	7.85[b]	6.90[c]	7.93[a]	0.001	0.258
General overall acceptability	7.13[d]	7.73[b]	7.63[c]	7.92[a]	0.001	0.117

Values are the means of triplicate determinations. Means along the rows with distinct superscript are substantially distinct from each other at α 0.05.

Key:
**Ctrl-Control (spontaneous fermentation) *C. gla- Candida glabrata *L. pla- Lactiplantibacillus plantarum *C. gla + L. pla- Candida glabrata + Lactiplantibacillus plantarum *P value- Probability value *SEM-Standard Error Mean*

4. CONCLUSION

The report on the physiological and proteomic response of *Lactiplantibacillus plantarum*LC03 and *Candida glabrata*YC02 isolated from fermented retted cassava to acid stress conditions at different pH concentration is presented. *Lactiplantibacillus plantarum*LC03 and *Candida glabrata*YC02 were able to survive the different acid stress conditions as well as show the expression of induced and repressed proteins. The identified proteins could be involved in the survival of the LAB and yeast to acid stress conditions, thereby enhancing the

improvement of mineral content and organoleptic properties of *Gari*, as well as a reduction of anti-nutrient contents of the product produced using *Lactiplantibacillus plantarum*LC03 and *Candida glabrata*YC02 as single and combined starters. Increased abundance of protein revealed the proteomic response of the LAB and yeast to acid stress conditions. Therefore, cassava fermentation can be improved by using stress-adapted organisms as starters.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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