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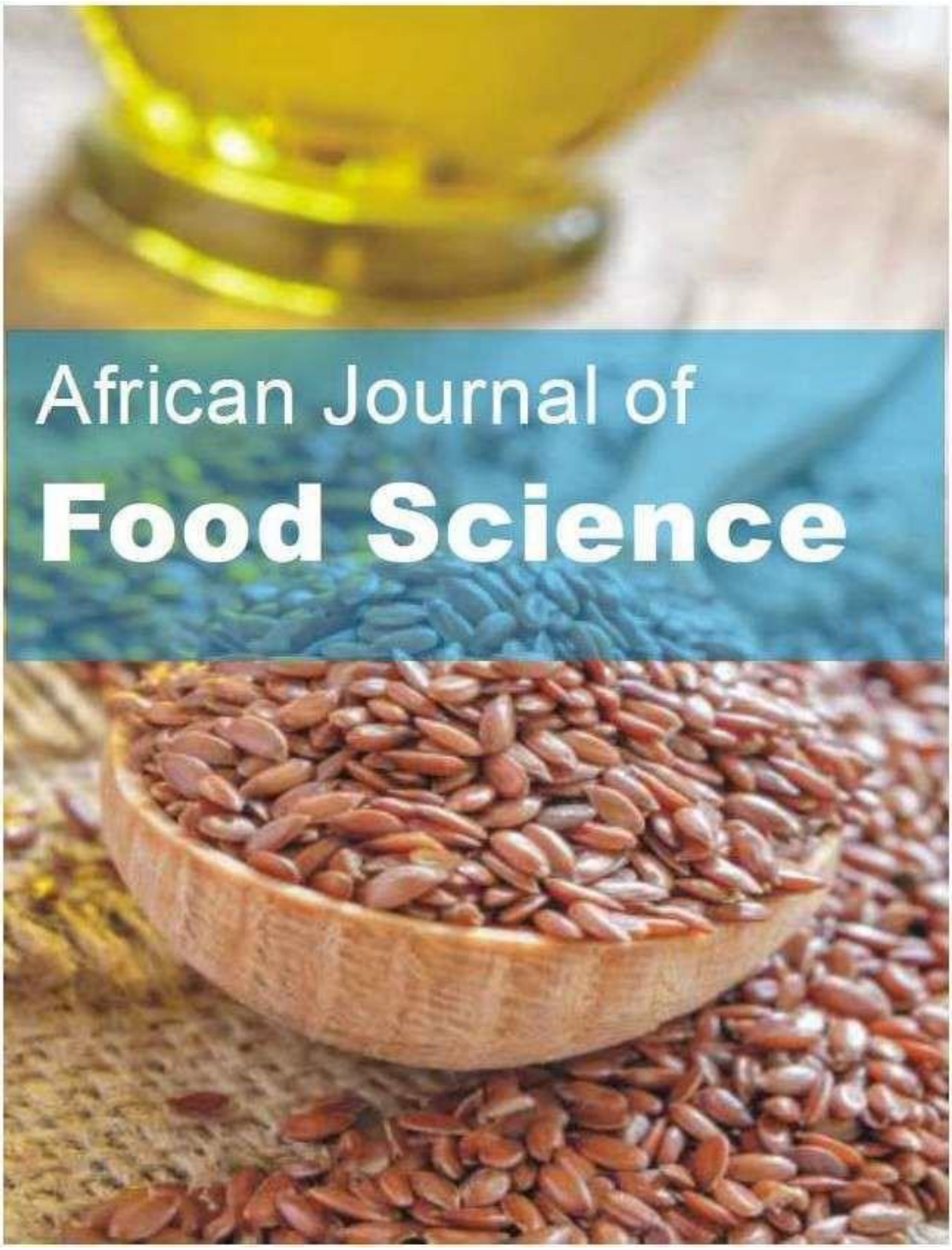

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African Journal of Food Science

Full Length Research Paper

Phytate degradation in composite wheat/cassava/sorghum bread: Effects of preincubation of *Pichia kudriavzevii* TY13 and presence of yeast extract

Serafina Lídia Vilanculos[1,2], Ulf Svanberg[2]* and Thomas Andlid[2]

[1]Departamento de Engenharia Química, Faculdade de Engenharia, Universidade Eduardo Mondlane, Maputo, Moçambique.
[2]Division of Food and Nutrition Science, Department of Biology and Biological Engineering, Chalmers University of Technology, Gothenburg, Sweden.

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Diet based on whole cereal flours is associated with a high prevalence of iron deficiency anemia and zinc deficiency in low/middle-income countries. Such flours contain high content of phytate that chelates minerals such as iron and zinc, making them unavailable for absorption by humans. To improve the mineral absorption, a phytate:iron molar ratio <1 and a phytate:zinc molar ratio <5 is needed to be achieved. This study aimed to improve the phytate degradation in composite wheat-cassava-whole sorghum flour bread by adding a phytase releasing yeast *Pichia kudriavzevii* TY13 in baking, preincubated or not, with addition of yeast extract. The phytate and mineral contents were measured by high-performance ion chromatography. Addition of *P. kudriavzevii* TY13 to the composite flour dough and fermentation for 2 h at room temperature resulted in a 98% phytate degradation. However, the same phytate reduction in the composite bread was achieved after 1 h fermentation at room temperature with addition of preincubated *P. kudriavzevii* TY13 plus yeast extract. Increasing the fermentation temperature to 30°C, the phytate content was equally low after fermentation for 1 h with *P. kudriavzevii* TY13 (preincubated or not) plus yeast extract. In conclusion, a faster reduction of phytate in composite bread was obtained by increasing the fermentation temperature, and addition of *P. kudriavzevii* TY13 (preincubated or not) with added yeast extract. The phytate to iron molar ratio was then 0.2 and the phytate to zinc molar ratio 0.6, which strongly indicates an improved bioavailability of both minerals from such a bread.

Key words: Phytate, phytase, *Pichia kudriavzevii* TY13, yeast extract, wheat flour, cassava flour, sorghum flour, bread making.

INTRODUCTION

Globally, about 2 billion people are affected by anemia and it is estimated that iron deficiency is the major cause of about half of all cases. In particular, iron deficiency is highly prevalent in many low-income countries (WHO, 2015) where cereals constitute the major staple food (Taylor et al., 1995; Tatala et al., 1998; Hurrell et al., 2002). The generally accepted explanation for this situation is that legume-cereal based meals are rich in phytate (myo-inositol hexaphosphate), an antinutrient whose phosphate groups strongly chelates divalent metal

ions of iron, zinc, and calcium making them unavailable for absorption by the human body (Hurrell et al., 2003; Prasad., 2013; Gupta et al., 2015).

Wheat bread is an important staple food in Mozambique, but the country only produces about 5% of its needs of wheat flour and therefore needs to rely on imported wheat at a high cost for the country (FAOStat, 2020). However, Mozambique produces other types of cereals and starchy roots that can successfully replace wheat flour to a large extent in bread making (Eduardo et al., 2014). In such local varieties of composite bread, it is essential to know how and when it is necessary to reduce the phytate content to improve mineral nutrition in Mozambique. In order to improve iron and zinc absorption, the phytate content needs to be enzymatically degraded by phytase. Phytases are a subgroup of phosphatases which catalyze a stepwise dephosphorylation of phytate to lower phosphoric esters of myo-inositol, releasing soluble inorganic phosphate and nonchelated minerals which becomes available for human intestinal absorption (Konietzny and Greiner, 2002). To improve iron absorption, it is recommended to degrade the phytate content to a phytate: iron molar ratio <1 (Hurrell et al., 2002) and for an improved zinc absorption the phytate:zinc molar ratio needs to be <15 (Nävert et al., 1985). In practice, this means that in cereal-based foods a phytate reduction of more than 95% needs to be achieved.

Several techniques have been applied to decrease the phytate content in cereal-legume based products. Examples are soaking, germination, fermentation, and addition of phytate degrading enzymes (phytases). These techniques are either based on activation of intrinsic phytases or by addition of exogenous phytase in the form of microorganisms synthesizing phytase or commercial phytases extracted from *Aspergillus niger*.

Soaking cereal flours of rye, maize, and sorghum at optimal conditions for phytase activity (55°C and pH 5) has been found to almost completely degrade the phytate content (Sandberg and Svanberg, 1991) and similar results were obtained in cereal-legume-based complementary foods soaked at optimal conditions for intrinsic cereal phytases (Egli et al., 2003). Soaking of milled wholegrains of sorghum and maize in de-ionised water without pH adjustment resulted in reduction of the phytate:iron molar ratio, however not to a level below one (Kruger et al., 2014). During fermentation, the production of organic acids (mainly lactic acid) causes a reduction in pH to levels at which the endogenous phytase in the cereal flours more efficiently degrades phytate. Studies have shown that a natural lactic acid fermentation of sorghum flours may reduce the phytate content up to 70% (Matuschek et al., 2001), and in combination with presoaking the sorghum flour an almost complete degradation of the phytate content was achieved (Svanberg et al., 1993). Kruger et al. (2012) succeeded to reduce the phytate content in a lactic fermented porridge of a genetically modified low phytate sorghum by 90%, however, still with a phytate:iron molar ratio >1.

In an attempt to optimize the baking conditions for phytate degradation by activation of intrinsic phytase, extended proofing time (2 h) at 37°C and pH adjusted to pH 4.5, Türk et al. (1996) succeeded to reduce up to 96% of the phytate content in whole wheat bread. Addition of a commercial microbial phytase from *A. niger* has been shown to completely degrade the phytate content in preparation of cereal-based complementary porridges (Hurrell et al., 2003). However, adding phytase from *A. niger* in baking of whole wheat bread has been less successful in reducing the molar ratio of phytate:iron to lower than one (Penella et al., 2008; Haros et al., 2001; Rosell et al., 2009). It would also be difficult in Mozambique to implement the strategy of adding a commercial phytase, since no such food grade product is up to now available in the country. It would thus be an advantage if the phytase could be naturally administered with the yeast in the baking process.

In the present study, a natural yeast *Pichia kudriavzevii* TY13 isolated from lactic fermented Tanzanian maize gruels (togwa) (Hellström et al., 2010) was applied with the aim to produce a composite bread with a phytate:iron molar ratio <1. Yeasts from the genera *P. kudriavzevii* has got GRAS status (Kurtzman et al., 2011) and the TY13 strain has shown a high capacity to synthesize and release phytase to the surrounding medium (Hellström et al., 2015; Qvirist et al., 2017). To achieve the aim, different strategies to improve the biosynthesis and release of phytase from *P. kudriavzevii* TY13 in the bread dough was applied, such as addition of growth promoting yeast extract, preincubation of the yeast and increased fermentation temperature and time.

MATERIALS AND METHODS

Ingredients

The ingredients used for the composite flours were wheat (*Triticum aestivum*) flour of lower extraction rate of 72%, which is a commercial white flour from Sweden (Frebago 1050 Bagerivetemjöl), cassava flour (*Manihot esculenta* Crantz) and non-tannin white whole sorghum flour (*Sorghum bicolor*) from Inhambane province in Mozambique (harvested in 2015). Cassava roots were peeled, washed, cut in pieces, and sun-dried for 4 days. During the drying period, the cassava pieces were flipped from time to time to ensure no mold contamination and then milled, packed,

*Corresponding author. E-mail: ulf.svanberg@chalmers.se. Tel: + 46 31 772 3819. Fax: +46 31 772 3830.

and stored. The sorghum grains were harvested, then washed, and damaged grains sorted out. After sun-drying for 2 days, the grains were milled at 100% extraction rate and finally packed and stored.

Bread making procedure

The bread was prepared by mixing 100 g of wheat flour, 50 g of cassava flour and 50 g of whole sorghum flour for 1 min in a kitchen aid (Artisan, Model 5KSM 150, USA), and then 135 mL of water and the rest of the ingredients were added (sugar 4 g, salt 2 g, baking yeast 4 g, margarine 6 g and ascorbic acid 0.1 g). The mixture was then blended for 2 min at speed level 2 followed by 3 min at speed level 4. At the mixing stage, 2 g of *P. kudriavzevii* TY13 was also added (with or without 2 g of yeast extract) or preincubated *P. kudriavzevii* TY13, and the pH of the dough was adjusted to 4.0 by adding a 20% lactic acid solution (~20 mL). Preincubation of 2 g *P. kudriavzevii* TY13 was done in 100 mL water of 30°C adjusted to pH 4.0 with lactic acid (with or without 2 g of yeast extract) and then the mixture was kept in a heating cabinet at 30°C for 1 h. The incubated yeast mixture was then added to the dry bread ingredients with an additional 35 mL of water. The dough was then covered with cotton cloth and left to ferment for 1 h at room temperature (~21°C) or at 30°C. After weighing, the dough was divided in four round shaped 80 g pieces and placed into baking pans and left to ferment for another 1 h and then baked as rolls for 8 min at 250°C in a kitchen oven. During the whole bread making process samples were withdrawn at all stages; after mixing, after fermentation (1 or 2 h) and at 30 min after baking and cooling. The withdrawn samples were placed into a plastic test tube and immediately frozen at -20°C. Finally, the samples were transferred to the freeze drier and dried for three days.

Phytate extraction and analysis

The phytate content was determined using an HPIC method developed by Carlsson et al. (2001). A milled freeze-dried sample of 0.5 g was extracted with 10 mL of 0.5 M HCl for 3 h at room temperature (22°C) under magnetic stirring. The extracts were frozen overnight, thawed, and centrifuged at 12000 rpm corresponding to 13400 × *g* for 5 min, and the supernatants were then decanted and 50 µL of supernatants injected and analyzed by HPIC with an Omni Pac PAX-100 (4 × 250 mm) analytical column and a PAX-100 (4 × 50 mm) guard-column (Dionex Corp., Sunnyvale, CA, USA). The separated inositol phosphates were detected after a post-column reaction with $Fe(NO_3)_3 \times 9H_2O$, using UV detection at 290 nm (Waters 486, tunable absorbance detector, Massachusetts, USA). The concentrations of phytate (*myo*-inositolhexa*kis*phosphate, $InsP_6$) were calculated using a standard curve created by external standards of $InsP_6$. All the reagents were of analytical grade (Sigma-Aldrich Co, St. Louis, MO, USA), and de-ionized water was used. The concentrations are presented on dry weight (DW) basis as the mean ± SD µmol/g.

Mineral extraction and analysis

For mineral extraction and analysis, the procedure according to Fredrikson et al. (2002) was followed. Approximately 250 mg of freeze-dried and ground sample was digested with 0.75 mL of nitric acid and 0.15 mL of concentrated hydrogen peroxide in Teflon vessels in an Ethos Plus microwave reaction system (model Multiwave PRO, Anton Paar Co., Ashland, VA, USA). After digestion to a transparent solution, samples were cooled to room temperature and diluted to a final volume of 10 mL with deionized water. Then 0.1 mL of ascorbic acid solution (20 g/L) was added to a sample volume of 0.9 mL to reduce Fe^{3+} to Fe^{2+} to avoid two peaks from the same element. A volume of 50 µL was injected and analysed by ion chromatography equipped with an IonPac CS5A column (250 × 4 mm, Dionex Corp., Sunnyvale CA) coupled with UV-vis detection at 500 nm, based on the formation of mineral complexes by pyridine-2,6- dicarboxylic acid in the mobile phase. Standard mineral solutions between 5 and 1000 ppb of iron and zinc were prepared by diluting an original concentration of 1000 ppm of the minerals in 0.05 M HCl and 2 g of ascorbic acid/L. For quality control in each batch of microwave digestion one vessel was used as a blank analysis, containing the nitric acid and peroxide hydrogen but not the samples.

Phosphate analysis

The content of dissolved inorganic phosphate (orthophosphate) in the yeast extract (Lot 105522, Scharlau Microbiology, Scharlab S.L., Spain) and the composite flour components was determined according to the HPIC method described by Qvirist et al. (2015). Each sample (~0.1 g) was mixed in 10 mL deionized water with pH adjusted to ~4.0 with added lactic acid, left to stand under agitation for 1 h and then centrifuged at 5000 g for 5 min. Aliquots of the supernatant were diluted in deionized water to achieve concentrations between 1 and 20 mg/L phosphate. The chromatograph consisted of a Dionex GS50 gradient HPLC pump equipped with a PAX-100 OmniPac guard and analytical column (Dionex Corp., Sunnyvale CA) and an anion self-regenerating suppressor (ASRS-300, 4 mm) at 50 mA (Dionex Corp.). The phosphate was eluted at a flow rate of 0.8 mL/min, using a gradient elution ranging from 2 to 49% of NaOH (0.2 M) with H_2O as counter eluent and a constant 2% isopropanol solution (50% in H_2O). Phosphate was detected using a conductivity detector (CD20, Dionex Corp.). Total run time for each sample was 35 min. Standard phosphate solutions of 1 to 10 µg/mL was used and the phosphate concentration was quantified by integrating the peak using the software Chromeleon (Dionex Corp).

Determination of dry matter

The dry matter was determined by a moisture balance encompassing the Precisa 310M mass balance and HA300 dryer (Precisa, Dietikon, Switzerland). A temperature of 70°C was used and initial sample weight was approximately 0.5 g.

Determination of pH

The pH was measured using Mettler Toledo MA 235 pH/Ion Analyzer. A 16 g dough piece was weighed and put into a flask tube and 8 g of water was added, then stirred using an electromagnetic plate (Retsch, Germany) and stirrer bar for 5 min. Finally, the pH was read using the pH meter probe.

Chemicals

The used chemicals were hydrochloric acid from Scharlau (Scharlab S.L. Spain), concentrated nitric acid from Fisher Chemical (Sweden), lactic acid from Scharlau (Scharlab S.L., Spain), deionized water, iron nitrate, ascorbic acid, peptone from (Bacto™, USA), glucose from Sigma Aldrich (Merck, Germany) and agar from Oxoid (Thermo Fisher, UK). The yeast extract was obtained from Scharlau Microbiology (Lot 105522, Scharlab S.L. Spain) that according to the manufacturer had a total nitrogen content of 10.9% corresponding to a crude protein content of 68.1 g/100 g out of which free amino acids constituted about 32 g/100 g. The total content of B-vitamins was about 140 mg/100 g. The phosphate content in the yeast extract measured by the HPIC method was 14.80 mg/g.

Table 1. Phytate and mineral content of the composite flours and bread per gram dry weight.

Type of flour	Phytate (µmol/g)	Fe (µg/g)	Phytate: iron molar ratio	Zn (µg/g)	Phytate:zinc molar ratio	Phosphate (mg/g)	Moisture (g)
Whole sorghum	11.1±0.67	85.5±0.02	7.2	16.2±0.02	44.4	1.32±0.06	0.12±0.05
Wheat	3.7±0.62	6.1±0.01	35.0	5.4±0.05	44.6	1.59±0.02	0.11±0.02
Cassava	3.5±0.71	3.8±0.05	52.1	4.6±0.07	50.0	1.23±0.03	0.11±0.07
Composite flour	5.5±0.09	26.3	10.8	8.2	40.4	1.46±0.07	0.15±0.03
Composite bread[a] (n=6)	1.04-0.07	23.7±1.60	2.6-0.2	7.8±1.10	8.7-0.6	-	0.14±0.08

[a)]Highest and lowest content of phytate in the composite breads.
Source: Authors

Preparation of yeast culture

A non-genetically modified strain of a wild-type *P. kudriavzevii* (TY13) (Qvirist et al., 2017) was used in the present study. The *P. kudriavzevii* TY13 was long term stored in 15% glycerol at -80°C and short term stored during the experimental periods on YPDA plates (yeast extract 10 g, peptone 20 g, glucose 20 g and agar 20 g in 1 L H_2O) at 4°C. As precultures, 5 mL of YPD in Falcon tubes were inoculated with TY13 from fresh YPD plates and incubated in a rotating carousel for 24 h at 30°C. The precultures were inoculated into yeast biomass production flasks: a set of 250 mL shake flasks each containing 200 mL YPD which were incubated for 24 h at 30°C under rotary shaking. To collect the yeast biomass, cultures were centrifuged at 4500 × *g* for 10 min using a Hereaus Multifuge (Kendro, Osterode, Germany), the supernatants were discarded, and the compressed yeast pellets were stored in a cold room (+4°C) until used within a few days.

Statistical analyses

Data are presented as mean values ± standard deviation of at least 3 replications. All statistical analyses were performed using SPSS (version 15.0, SPSS Inc., Chicago, IL) software. Mean values were compared by analysis of variance, and determination of significant differences between variables was made with Tukey´s HSD post hoc multiple range test. Differences were considered to be significant at $p<0.05$.

RESULTS

Phytate and mineral content

The cassava flour had a lower content of minerals, 3.8 µg/g for iron and 4.6 µg/g for zinc, compared with the wheat and much lower compared with whole sorghum flours (Table 1). In the composite flour with a mixture of the three flours, thus a significantly higher mineral content was obtained, for iron 26.3 µg/g and zinc 8.2 µg/g. The inorganic phosphate content in the three composite flours was for wheat 1.59 mg/g, for sorghum 1.32 mg/g, and for cassava 1.23 mg/g. In the yeast extract the phosphate content was significantly higher, 14.80 mg/g.

The highest phytate content was found in whole sorghum flour (11.1 µmol/g) with significantly lower content in wheat (3.7 µmol/g) and cassava flours (3.5 µmol/g). The phytate to iron molar ratios were high in all the flour types including the composite flour, from 7 in the whole sorghum flour to 52 in the cassava flour and about 11 in the composite flour. Moreover, the phytate to zinc molar ratios were also high in the flours, ranging between 40 and 50.

Phytate degradation at different stages in the baking process

Figures 1 to 4 show the phytate content at different stages in the baking process; after the mixing stage, after fermentation for 1 and 2 h at either room temperature or 30°C, and in the final composite bread. The pH was adjusted to 4.0 at the mixing stage and 2.0 g (wet weight) of *P. kudriavzevii* TY13 was added, preincubated or not, and with and without the addition of 2.0 g yeast extract. A composite bread without the addition of *P. kudriavzevii* TY13 was used as a reference bread.

Dough mixing step

Already at the mixing stage at room temperature, there was a significant reduction of the phytate content, between 27 and 42% in comparison with the phytate content in the composite flour. There was, at this stage, no difference between the doughs with and without added *P. kudriavzevii* TY13 (Figures 1 and 2). There was an additional degradation in the doughs with added preincubated *P. kudriavzevii* TY13 ($p<0.05$), with a phytate reduction up to 56% observed when mixing was done at room temperature (Figure 2). However, there was no further enhanced phytate degradation by adding yeast extract in the preincubation with *P. kudriavzevii* TY13. Dough mixing at the higher temperature of 30°C (Figures 3 and 4) resulted in phytate reductions between 38 and 56% with addition of *P. kudriavzevii* TY13.

Dough fermentation step

The fermentation step resulted in a continued phytate degradation, and both preincubation of *P. kudriavzevii* TY13 and addition of yeast extract had a significant effect. After fermentation for 1 h, both at room

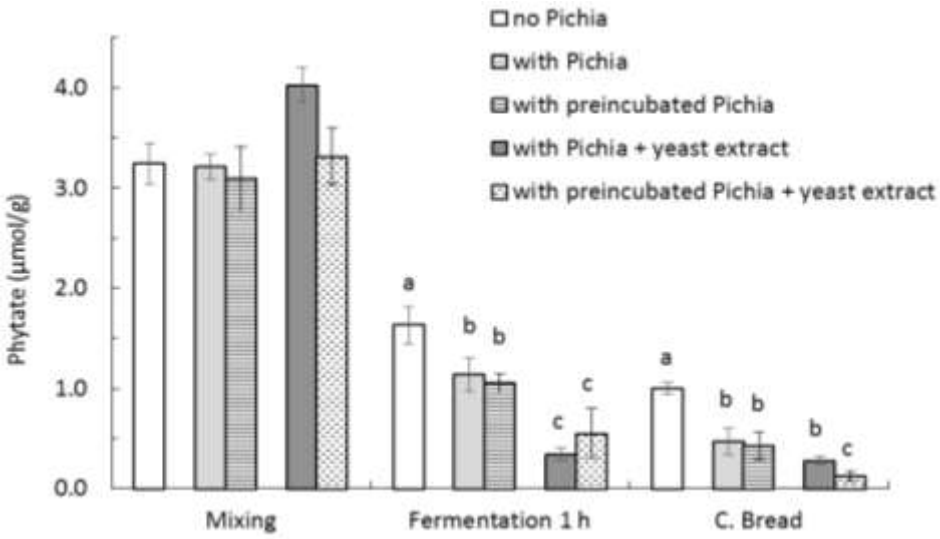

Figure 1. Phytate content during baking of composite bread with pH at 4.0, 1 hour fermentation at ambient temperature with and without 2.0 g of *P. kudriavzevii* TY13 (preincubated or not) and addition of yeast extract (Means ± S.D., n = 3). Samples within each treatment showing a different letter (a-c) are significantly different $p<0.05$.
Source: Authors

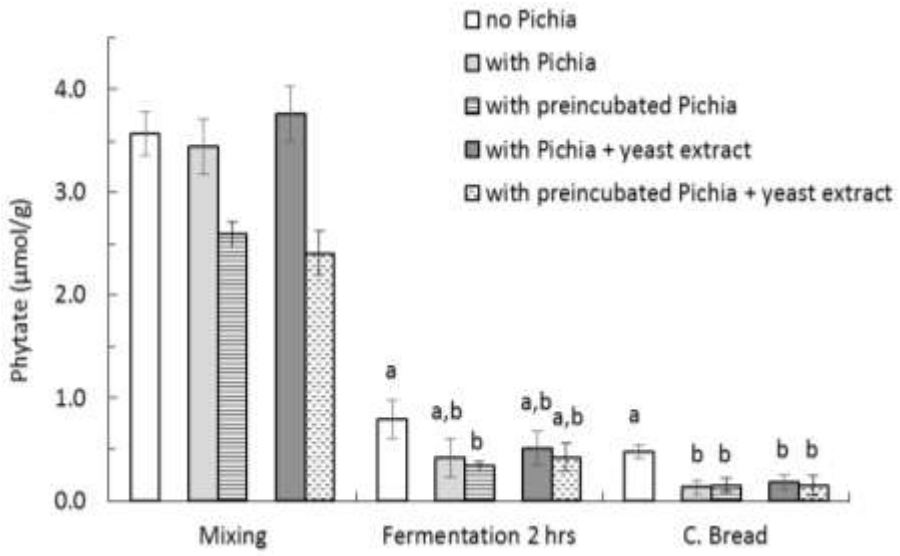

Figure 2. Phytate content during baking of composite bread with pH at 4.0, 2 h fermentation at ambient temperature with and without 2.0 g of *P. kudriavzevii* TY13 (preincubated or not) and addition of yeast extract (Means ± S.D., n = 3). Samples within each treatment showing a different letter (a-b) are significantly different $p<0.05$.
Source: Authors

temperature and 30°C, with added *P. kudriavzevii* TY13, the phytate content was about 1.0 µmol/g and significantly lower, about 0.40 µmol/g with addition of yeast extract (Figures 1 and 3). There was an additional phytate reducing effect by adding preincubated *P. kudriavzevii* TY13 at the higher fermentation temperature (30°C), the phytate content being 0.58 µmol/g compared with 0.94 µmol/g ($p<0.05$) in the dough with non-preincubated *P. kudriavzevii* TY13 (Figure 3). By increasing the fermentation time to 2 h at 30°C, the lowest phytate content of 0.09 µmol/g was achieved in the dough with preincubated *P. kudriavzevii* TY13 and no additional effect was at these conditions observed with addition of yeast extract (Figure 4).

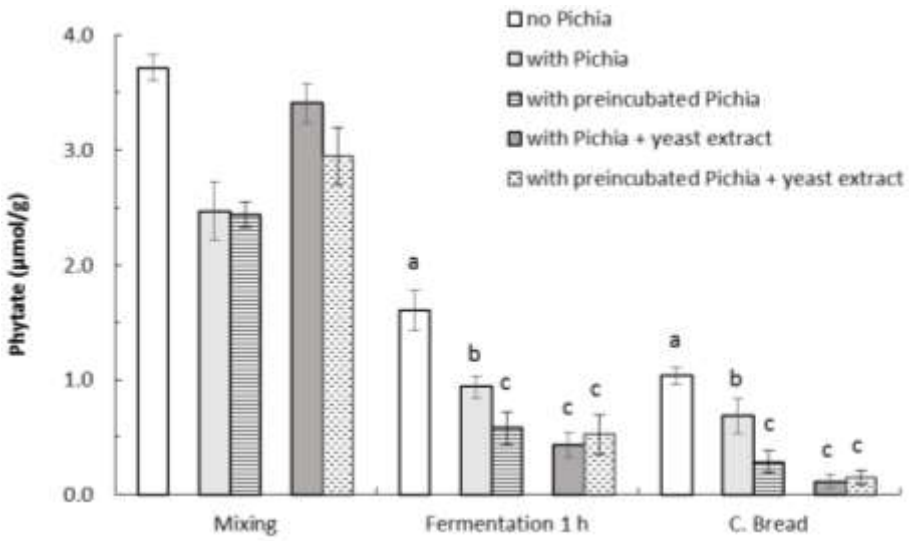

Figure 3. Phytate content during baking of composite bread with pH adjusted to 4.0, 1 hour fermentation at 30°C with and without 2.0 g of *P. kudriavzevii* TY13 (preincubated or not) and addition of yeast extract (Means ± S.D., n = 3). Samples within each treatment showing a different letter (a-c) are significantly different $p<0.05$.
Source: Authors

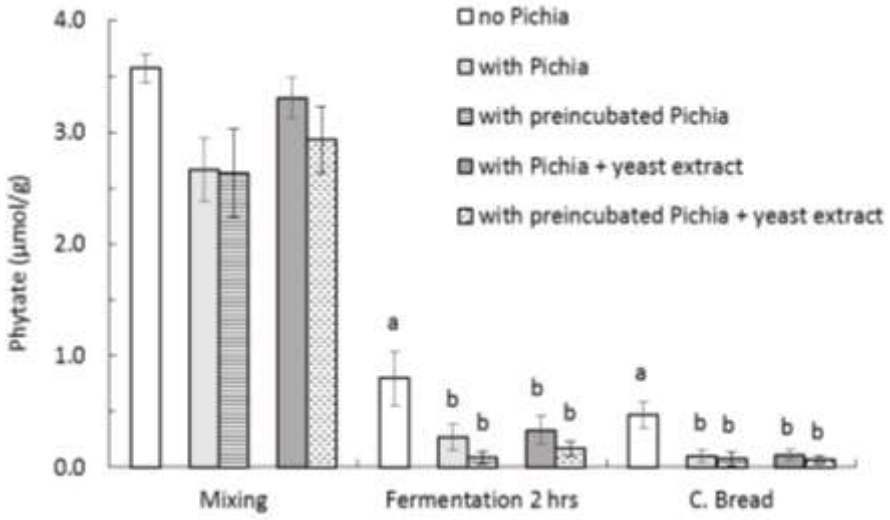

Figure 4. Phytate content during baking of composite bread with pH adjusted to 4.0, 2 hours fermentation at 30°C with and without 2.0 g of *P. kudriavzevii* TY13 (preincubated or not) and addition of yeast extract (Means ± S.D., n = 3). Samples within each treatment showing a different letter (a-b) are significantly different $p<0.05$.
Source: Authors

In composite bread

By increasing the fermentation time, additional degradation was achieved in the composite breads with *P. kudriavzevii* TY13, with and without yeast extract. After 2 h fermentation at either room temperature or 30°C, the phytate content was consistently low and reduced to between 0.07 and 0.18 µmol/g (Figures 2 and 4). This corresponds to a phytate:iron molar ratio of 0.2 to 0.4 and a phytate:zinc molar ratio of 0.6 to 1.5.

However, the same low phytate content was achieved in the composite bread fermented for 1 h at 30°C with *P. kudriavzevii* TY13, preincubated or not plus addition of yeast extract (Figure 3) as well as in the composite bread fermented for 1 h at room temperature with preincubated *P. kudriavzevii* TY13 and additional yeast extract (Figure 1).

DISCUSSION

Phytate and mineral content

Cassava has generally a lower amount of minerals than cereals and a major part of the minerals are found in the root peels (Montagnac et al., 2009). The highest content of phytate and minerals was in our work obtained in whole sorghum flour as a result of the bran fraction, which is rich in phytate as well as minerals (Hurrell, 2003). Since one main function of phytate in plant seeds is mineral storage, this was expected. On the other hand, the wheat and cassava flours used in our study had lower iron and zinc contents because of low extraction rate for the wheat (not fortified) and exclusion of peels for the cassava roots during flour preparation. The phytate content of the wheat and cassava flours were at the same level as previously reported (Lazarte et al., 2015). Of the bread flour ingredients, the whole sorghum flour had the lowest phytate to iron molar ratio of 7.2 due to its high content of iron, whereas the composite flour used for the baking experiments had a ratio of 10.8. As this is many-fold higher than 1, composite bread products prepared from such flours are expected to result in low intestinal absorption of iron in humans (Hurrell, 2004). However, this may be improved by strategies to reduce the phytate content during the baking process, to obtain phytate to iron molar ratios below 1. This would sufficiently decrease the relative fraction of chelated non-available Fe in favor of non-chelated Fe, available for intestinal uptake. The same initial problem was true for zinc; phytate to zinc molar ratio was higher than 15, demonstrating low zinc absorption (Nävert et al., 1985).

Phytate degradation at different stages in the baking process

The baking procedures were based on strategies to allow for a higher phytate degradation in the composite breads, that is, increased fermentation time and temperature, and the addition of the non-conventional yeast *P. kudriavzevii* TY13, in prior work shown to be superior in phytate degradation (Hellström et al., 2010, 2012, 2015; Qvirist et al., 2017). The yeast was added at the mixing stage, with or without preincubation, and addition of yeast extract. The rationale behind adding yeast extract was based on our earlier work demonstrating that addition of yeast extract to a synthetic yeast medium (YNB) increases phytase release to the surrounding (assessed as activity in cell free supernatant after centrifugation). In those experiments, it was shown that the observed increased activity was not a result of increased growth rate (compared with same medium without yeast extract) but by increased secretion of non-cell-bound phytase (Hellström et al., 2015).

In the present work, there was a significant phytate degradation at the mixing stage at room temperature, and especially so with addition of preincubated *P. kudriavzevii* TY13. With a higher temperature in the mixing stage (30°C) there was a significantly higher phytate degradation in the doughs with added *P. kudriavzevii* TY13, on average 50% compared with the doughs without addition of *P. kudriavzevii* TY13, ~35%. Thus, the degradation observed at the mixing stage suggests that prevailing conditions resulted in (i) activation of intrinsic flour phytases and (ii) production and release of active extracellular phytase by *P. kudriavzevii* TY13.

The composite doughs with *P. kudriavzevii* TY13 fermented for 1 h at room temperature showed a higher phytate reduction (79%) than the composite doughs without *P. kudriavzevii* TY13 (70%). This indicates that the released active phytase from the *P. kudriavzevii* TY13 contributed to the phytate degradation that has been achieved by the intrinsic phytases in the composite flours as earlier has been reported by Türk et al. (1996), Egli et al. (2003), and Vilanculos and Svanberg (2021). By increasing the fermentation time to 2 h, the composite doughs with *P. kudriavzevii* TY13 still showed higher phytate degradation (92%) than the composite dough with no *P. kudriavzevii* TY13 (85%).

In the composite breads with added *P. kudriavzevii* TY13 fermented for 1 h at room temperature, the trend was the same as in the doughs after fermentation; the phytate reduction was higher (92%) in the composite breads with *P. kudriavzevii* TY13 (preincubated or not) than in the breads without added *P. kudriavzevii* TY13 (80%). Adding yeast extract resulted in a further improved phytate degradation, 95%, that increased to 98% ($p<0.05$) with preincubated *P. kudriavzevii* TY13 (Figure 1). This effect could be explained by the higher production and release of extracellular phytase after preincubation of *P. kudriavzevii* TY13 plus yeast extract as previously shown by Hellström et al. (2015). Yeast extracts contain a mixture of amino acids, peptides, vitamins and carbohydrates that promotes growth of microorganisms such as yeast and fungi as well as production of secondary metabolites including phytase (Kłosowski et al., 2018; Li et al., 2011; Sasirekha et al., 2012; Sørensen and Sondergaard, 2014). With respect to TY13, we have shown that the main effect is on increased biomass specific phytase activity released to the medium, rather than on general increased growth. Among tested nitrogen sources (yeast extract, meat extract and peptone from casein) in cultivation of lactic acid bacteria, the highest cell concentration and phytase biosynthesis was achieved in yeast extract cultivation (Raman et al., 2019). The same has been found true for our strain TY13 (Hellström et al., 2015).

However, high phosphate conditions are known to repress the synthesis of acid phosphatases and phytases, while limiting phosphate conditions result in their expression. A sharp decline in phytase production of *A. niger* was observed even at a phosphate concentration

of 5 mM in the growth medium with no production at 10 mM and above (Vats and Banerjee, 2002). The yeast variety *P. kudriavzevii* TY13 has previously been shown to produce extracellular phytase in the presence of inorganic phosphate up to 5 mM concentration in lactic fermented Tanzanian maize gruels (Hellström et al., 2012) and in high phosphate synthetic medium (Qvirist et al., 2017). In conventional baker's yeast, phytase activity is strongly repressed at such levels of phosphate (Andlid et al., 2004). In our study, the free inorganic phosphate concentration in the composite dough could be estimated to about ~23 mM and with addition of yeast extract to about 26 mM, and still production and release of phytase by *P. kudriavzevii* TY13 was observed. At the preincubation step of *P. kudriavzevii* TY13 plus addition of 2 g of yeast extract the inorganic acid phosphate concentration is about 3 mM which indicates no phosphate induced inhibition on phytase synthesis and secretion by *P. kudriavzevii* TY13.

By increasing the fermentation time to 2 h, however, all composite breads with *P. kudriavzevii* TY13 degraded the same high amount of phytate (98%) (Figure 2). Obviously, addition of yeast extract had no additional effect when fermentation time was prolonged at ambient temperature.

However, at a fermentation temperature of 30^{o}C, close to optimal growth temperature for the yeast, the addition of yeast extract increased phytate degradation already after fermentation for 1 h. Most likely the yeast extract somehow induced an increased synthesis and release of phytase from the *P. kudriavzevii* TY13 (Hellström et al., 2015) at a higher temperature. Whether this is a result of a general higher amount of growth factors that promote yeast growth, or if yeast extract contains specific phytase biosynthesis promoting compounds is not known. No additional reduction of the phytate content was observed in the composite breads fermented for 2 h at 30°C (compared with 1 h), suggesting that all phytate available for the yeast phytase was degraded already at 1 h, where the reduction was close to 100%.

Conclusion

Our results show that optimized fermentation temperature and pH in the baking of composite flour bread with added phytase releasing yeast *P. kudriavzevii* TY13 and yeast extract as growth and/or phytase production/release promotor improves the phytate degradation in the final bread. A unique property of TY13 is its capacity to produce and release active phytase in presence of inorganic phosphate which is ubiquitous in most foods including the composite flours in this study. Adding the *P. kudriavzevii* TY13 with and without yeast extract directly to the dough at the mixing stage and fermentation for 2 h at room temperature resulted in an almost complete phytate degradation. The same effect was obtained in the composite bread with 1 h fermentation time at 30°C and addition of *P. kudriavzevii* TY13 (preincubated or not) with yeast extract. This corresponds to a phytate to iron molar ratio of 0.2 and a phytate to zinc molar ratio of 0.6. Both ratios are low enough to allow an improved absorption of both minerals in humans. Finally, the practical applications of our findings are that it is possible to obtain a composite flour bread with nearly zero phytate content by addition of *P. kudriavzevii* TY13 in combination with ordinary baker's yeast plus yeast extract and baking off under optimal conditions that are possible to achieve in commercial bakeries.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Full Length Research Paper

Production of maize-soybean complementary foods fortified with crayfish, bonga fish and carrot flours rich in essential nutrients

Emeka Felix Okpalanma[1*], Ekpeno Sunday Ukpong[1], Onuora Charles Chude[2] and Chizoba Rejoice Abah[3]

[1]Department of Food Science and Technology, Madonna University, Akpugo, Enugu State, Nigeria.
[2]Department of Food Science and Technology, Chukwuemeka Odumegwu Ojukwu University, Igbariam, Anambra State, Nigeria.
[3]Department of Food Science and Technology, Nnamdi Azikiwe University, Awka, Anambra State, Nigeria.

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Blends of maize-soyabean complementary foods were fortified with foods rich in calcium, iron, zinc and vitamin A. Crayfish, bonga fish, and carrot that were processed into flours separately and blended in ratios (2:1:1 w/w) to produce food fortificant. Maize flour, soyabean flour and fortificant were mixed in the ratio of 70:30:0, 60:30:10 and 50:30:20 (% w/w dry basis), respectively to obtain three blends of complementary food. Standard chemical methods were used to determine the blends' proximate composition, mineral, vitamin A and anti-nutrientional factors. The unfortified blend contents of calcium, iron, zinc and vitamin A were at level of 417.37, 9.15, 6.20 mg/100 g and 387.67 µg RE/100 g, respectively but increased in the fortified blends to the range of 560.45 to 620.12 mg/100 g, 12.43 to 14.25 mg/100 g, 8.72 to 10.67 mg/100 g, and 550.13 to 710.25 µg RE/100 g. Fortified blends formula had micronutrients significantly higher ($p<0.05$) than the proprietary formula, Cerelac. The blends had 4.03 kCal/g average energy content, 12-19% protein-energy ratio and 25-28% energy from lipids. The proposed fortification levels are based on a daily ration size of 65 g for infants aged 9 to 11 and children aged 12 to 23 months. Feeding 65 g of the diets to infants aged 6 to 11 months will meet the 200, 300 kCal/day and 350 µg RE/day Recommended Nutrient Intakes (RNI) for energy and vitamin A and will be adequate for the minerals (calcium and zinc) requirement of children aged 12 to 23 months.

Key words: Complementary foods, maize, soybean, crayfish, carrot, *bonga* fish.

INTRODUCTION

Complementary feeding has been recommended to start at 6 to 23 months of infants' age when they are fed breast milk, other soluble foods and liquids that are enough to meet their nutritional requirements, while continuing breast feeding beyond two years (Campoy, 2018). Balanced nutrition administered to infants and children will definitely give rise to the development of each child's full human potential. Inadequate nutrition at early stages results in long term growth and health impairment. Early under nutrition or nutritional defects during the first 2 years of life are causes of impairment of infants and children's intellectual capabilities, stunting and wasting

*Corresponding author. E-mail: felixokpalanma@yahoo.com. Tel: +2348033924371.

(Srivastava and Chaturvedi, 2020). Available records had it that stunting affected 149 million children under 5 worldwide (Dulal et al., 2017). Also 6.7% of children under 5, or 45.4 million are affected by wasting which is the most destructive form of malnutrition (WHO/UNICEF, 2021). During the breastfeeding and complementary feeding period, essential nutrients should be fed to children to reach their full physical and cognitive potentials, with the attendant benefits that last into adulthood (Arikpo et al., 2018; Owais et al., 2017). Panjwani et al. (2017) reported that 45% of deaths in children under 5 are consequences of micronutrient malnutrition which is the major cause of ill-health in this age group.

Iron and vitamin A deficiencies present major nutritional deficiencies in the world today of greatest significance in public health management. Undernourishment affects more than 800 million people in the world and it had been reported that between 1.5 and 2 billion people have chronic and severe micronutrient deficiencies (MND), mostly deficiencies in the minerals iron, selenium, calcium, zinc and vitamins A and folate (Beal et al., 2017; FAO, 2020). The instruments of fortification, supplementation, food-based approaches with dietary diversification have been used to resolve issues associated MND. Supplementation failed to address the root cause of the MND despite its cost effectiveness. Food-based approaches have therefore recently been suggested to offer over a long term, more practical and cheaper means to address MND, given its capacity in providing the opportunity to target a larger segment of poorer population (Regan et al., 2015).

Experts recently agreed that greater emphasis should be directed to the food approach and consumption of large quantities of micronutrient-rich foods (Guamuch et al., 2014). Therefore, one of the major aims of this study is to use blends of pre-treated maize-soybean flours with good balance of nutrients and fortification of the blends with foods (crayfish, bonga fish and carrot flours) rich in the nutrients to enhance the densities of vitamin A, calcium, zinc and iron that would meet the recommended daily allowance (RDA) for infants and young children. Also part of the study is to compare the nutrients composition of the complementary food blends with Cerelac - a product of Nestle (Nigeria) PLC so as to assess potentials of the complementary foods in addressing issues of micronutrient deficiencies (MND) prevalent in third world countries especially Nigeria.

MATERIALS AND METHODS

Maize *(Zea mays* L.), soyabeans (*Glycine max*), carrot (*Daucus carota*), *bonga* fish (*Ethmalosa fimbriata*) and crayfish *(Macrobrachium* species) were purchased from Ogbate market in Enugu, Nigeria. The foodstuffs were used to formulate three complementary foods composite blends in this study. Also, Cerelac (a product of Nestle Nigeria PLC), was purchased from the same market and used for comparison purposes (Table 2).

Preparation of germinated maize and soybean grains

Germination was done by the modified method of Ren et al. (2017). Maize and soybean seeds were disinfected by soaking in 70% ethanol solution for 15 min at room temperature after cleaning by hand to remove foreign materials. The ethanol was washed out of the seeds with tap water and distilled water and later soaked in distilled water (1:5, w/v) for 12 h at room temperature (~30°C). The seeds were placed between thick layers of jute bag after the water was drained off and germination lasted for 4 days in the dark. Fresh distilled water was used every day to moisten the seeds. The germinated seeds coats were removed. The decoated-germinated beans were subjected to 10 min steaming and subsequently cooled to room temperature and dried at 50°C in a Gallenkamp oven (ModelIH-150; Gallenkamp, England) and later milled into flour using a Bentall attrition mill (Model 200 L090). The flour was stored in polyethylene bags at 4°C for further analysis.

Preparation of carrot, bonga fish and crayfish flours

Tap water was used to wash the carrots which were air dried for 48 h after processing with kitchen grater. Crayfish and bonga fish (bony fish) were milled and sun-dried on a hot day and high breeze for 12 h.

Preparation of composite flours

The formulations of three complementary foods composite flours and fortificants were as follows:

Blend 1: Maize: Soyabean: food fortificant (70:30:0 %w/w)
Blend 2: Maize: Soyabean: food fortificant (60:30:10 %w/w)
Blend 3: Maize: Soyabean: food fortificant (50:30:20 %w/w)
Food fortificants: Crayfish: Bonga fish: Carrot. 2:1:1 (w/w) ratio

Each of the three complementary foods composite flours mixtures was separately milled into a homogenous powder in a 2 L mistral grinder and stored in containers that are airtight awaiting analyses. The Nestle Cerelac was used as control in comparing the nutrient levels of the composite blends against quantities of nutrients present in the estimated 65 g (dry weight) daily intake size of local weaning food by 6 to 11 month old infants. Thereafter, the blends nutrient densities were compared with Recommended Nutrient Intakes (RNIs) to assess the local diets compliance with the nutrient recommendations (Solomon, 2005).

Analysis

The proximate composition (moisture, crude protein, fat, crude fibre and ash) of the fortified maize-soyabean blends were determined by AOAC (2009) methods. Triplicate analysis was carried out to obtain a mean value for each nutrient. Carbohydrate content was determined by subtracting the sum of moisture, protein, fat, ash and crude fibre percentage from 100.

The energy value was determined by Atwater conversion factor (Nyahabeh et al., 2020) as follows:

$$Energy\ value\ (kCal/100\ g) = 4 \times protein + 9 \times fat + 4 \times carbohydrate$$

The oxalate contents of the fortified complementary food blends were analysed using the spectrophotometric (Genway 6305, England) method of Fassat (1973) as modified by Mishra et al. (2017). The method of Price et al. (1980) as described by Abidemi (2013) was used to analyse the tannin content. Also, the analysis of phytate was according to the method of Latta and Eskin (1980) as

Table 1. Proximate composition of maize -soybean complimentary food blends and Nestle Cerelac.

Nutrient	Blend 1	Blend 2	Blend 3	Cerelac
Crude protein (%)	13.17^{a}±0.05	16.25^{b}±0.02	19.30^{c}±0.01	15.85^{b}±0.31
Crude Fat (%)	11.55^{b}±0.40	12.33^{a}±0.51	12.60^{a}±0.46	9.63^{c}±0.42
Crude fibre (%)	4.10^{a}±0.02	5.37^{b}±0.05	6.46^{c}±0.01	3.25^{d}±0.11
Ash (%)	2.75^{a}±0.03	3.95^{b}±0.02	4.81^{c}±0.02	2.80^{a}±0.01
Moisture (%)	5.41^{b}±0.10	5.22^{b}±0.20	4.31^{a}±0.10	2.75^{c}±0.02
Carbohydrate (%)	56.88^{b}±2.41	52.52^{c}±2.12	65.68^{a}±3.21	63.02^{a}±1.32
Energy(kCal/100 g)	403.49^{a}±15.67	400.68^{a}±13.45	412.79^{b}±10.43	408.71^{a}±12.31

Values are means ± SD of triplicate determinations. Means with the same superscript along each row are not significantly different (p<0.05).
Source: Authors

Table 2. Calcium, iron, zinc (mg/100 g) and vitamin A (µg RE/100 g) content of maize -soybean complimentary food blends.

Micronutrient	Blend 1	Blend 2	Blend 3	Nestle Cerelac
Calcium	417.34^{a}±0.19	560.45^{b}±0.52	620.12^{c}±0.21	419.41^{a}±0.01
Iron	9.15^{a}±0.02	12.43^{b}±0.05	14.25^{c}±0.02	11.23^{d}±0.01
Zinc	6.20^{a}±0.12	8.72^{b}±0.23	10.67^{c}±0.13	7.81^{d}±0.
Vitamin A	387.67^{a}±0.18	550.13^{b}±0.21	710.52^{c}±0.10	400.42^{d}±0.11

Values are means ± SD of triplicate determinations. Means with the same superscript along each row are not significantly different (p<0 .05).
Source: Authors

modified by Sivakumaran and Kothalawala (2018). The vitamin A content in the fortified complementary food blends was assayed according to the method of Arroyave et al. (1982). Minerals' (iron, zinc and calcium) content was assayed using Atomic Absorption Spectrometer (AAS) (model 210 VPG) according to the method described by the AOAC (2009).

Statistical analysis

Each experiment was performed in triplicate. Statistical Package for Social Science (SPSS) version 17 was used for the Analysis of Variance. Significantly different means were separated using Least Significant Difference test (LSD) at p<0.05.

RESULTS AND DISCUSSION

Composition of maize-soybean complementary blends

Table 1 shows the proximate composition of maize-soyabean complementary food (CF) blends. The blends energy content ranged from 400.68 to 408.71 kCal/100 g, with average of 4.04 kCal/g energy density. Codex Alimentarius Guidelines (FAO/WHO, 2017) for formulated supplementary foods recommended energy density of at least 400 kCal/100 g of dry food for older infants and young children. Significant difference (p<0.05) was not observed between the energy content of the fortified blends and non fortified blends. However, for age groups 6-8 months, 9-11 months and 12-23 months, the total energy requirements are 615, 686, and 894 kCal/day, respectively. Also, for infants in developing countries that lack adequate breast milk intake, the energy need from CFs was expected to increase from 200 to 300 kCal/day and 550 kCal/day at 6-8 months, 9-11 and 12-23 months (Table 3), respectively (WHO/UNICEF, 1998; Dewey and Brown, 2003).

A proposal of fortification levels based on a single daily ration size of 65 g (Table 3) for the age groups was made. It follows that the consumption of 262 kCal/day (65 g of dry product of blend 2) by infants, 9-11 months, and children 12-23 months will amount to 88.5 and 48% of their energy intake from complementary foods. Also, the observed inverse relationship between the age and proportion of energy requirements that would be met is attributed to the fact that a greater proportion of complementary foods provided from the family diet would be consumed gradually by the child (Abeshu et al., 2016). Infants have only limited gastric capacity to consume adequate quantity of food, hence the recommendation by the World Health Organisation that they be fed with high nutrient density diets (Stephenson et al., 2017).

The crude protein contents of fortified CF blends vary from 13.17 to 19.30 mg/100 g. Results showed

Table 3. Estimated Amounts of Nutrient Provided by the proposed daily ration size of 65 g of the complementary food blends compared with 65 g of Nestle Cerelac and Recommended Nutrient Intakes (RNI[a]).

Nutrient	RNI[a]						
	6-8 months	9 - 11 months	12 -23 months	Blend 1	Blend 2	Blend 3	Nestle Cerelac
Energy(kcal)	200	300	550	265.58	262.36	260.00	267.4
Crude protein (g)	9.1	9.6	10.9	8.55	10.55	12.46	9.47
(% total energy)		8 - 15 %		(12.90)	(16.11)	(19.26)	(14.52)
Crude fat (g)	9	10	NA	7.50	8.00	8.13	4.34
(% total energy)	19%	24%	28%	(25.44)	(27.46)	(28.13)	(14.54)
Carbohydrate (g)		NA		41.24	37.24	34.37	44.48
(% total energy)		62 - 70 %		(62.05)	(56.80)	(52.87)	(66.53)
Calcium (mg)	525	525	350	271.27	364.29	403.00	272.62
Iron (mg)[d]	11	11	6	5.94	8.07	6.93	5.07
Zinc (mg)[f]	4.1	4.1	1.1	2.66	4.20	5.14	3.38
Vit A (µg RE)	350	350	400	251.98	357.58	461.83	260.27
Moisture (g)		<5		3.51	3.39	2.80	1.79
Ash (g)		<3		1.79	2.56	3.12	1.82
Crude fiber (g)		<5		2.67	3.49	3.13	1.79

RNI[a]: Recommended Nutrient Intakes, [d]Assuming medium bioavailability (10%), [f]Assuming moderate bioavailability (30%), NA: Not available. WHO (1998); Dewey and Brown (2003); Joint FAO/WHO Expert Consultation (2002)[f].
Source: Authors

significantly ($p<0.05$) higher values than unfortified blend. The increase in protein content followed with incremental addition of fortificants. This may be due to protein contributions from bonga fish and crayfish in the fortificant (FAO/WHO, 2017).

The percentage total energy of 12.90, 16.11 and 14.52% (blends 1, 2 and cerelac) (Table 3) contribution from protein in 65 g CF agreed with the 8-15% recommendation (Alvisi et al., 2015). The reported Protein-Energy (PE) ratios of our CF blends are therefore adequate.

The fortified blends fat content (12.33-12.60 g/100 g) were 6.75 to 9.10% more than the fat in unfortified blend. The observed high values were probably contributions from fortificants. The fat contents in 65 g of the formulated CF (7.5-8.13 g) were comparatively higher than that from commercial formular, Cerelac (4.34 g). The addition of oil-dense soya beans in the local blends could be responsible for the higher values. The percentage of energy as fat from the proposed 65 g formulated CFs is 25.44 to 28.13% (Table 3). This is in agreement with recommendation of 24 and 28% energy sourced from fat for infants and children aged 6 to 11 months, and 12 to 23 months, respectively (WHO/ UNICEF, 1998; Dewey and Brown, 2003). The energy, essential fatty acids, and fat soluble vitamins (A, D, E, and K) for infants and young children are derived from dietary fats (Abeshu et al., 2016).

The fortified blends fibre content (5.37 to 6.46 g/100 g) was 31.0 to 57.6% higher when compared with the unfortified blend. The fiber content increases with level of fortificants added. This may be due to carrot flour which contains relatively large amount of cellulose as fibre. Bioavailability of micronutrients during infancy is encouraged by the reduction of use of large quantities of pulses, whole grain cereals and nuts since they could cause a low-energy diet (Caballero et al., 2005). The level of fibre obtained in this study is within the standard which must not exceed 5 g/100 g of food. The low fibre content could result from the use of dehulled raw materials in the formulation.

The fortified blends carbohydrate content varied from 52.52 to 56.88 mg/100 g (Table 1). The values were 10.8 to 20.1% lower than unfortified blend. The carbohydrate content in the proprietary formula (44.8 g) is higher than the CF blends (34.37- 41.24 g). This could be as a result of increases in protein, fat, ash and fiber contents resulting from CF blends fortification.

The fortified blends ash content varies from 3.95 to 4.81 g/100 g and 43.63 to 70.90% higher when compared with the unfortified blend. The increase which is probably an indication of presence of high quantity of minerals, especially the macro minerals might be attributed to the fortification effect. The ash content (Table 3) in the proposed 65 g CF blends (2.56-3.12 g) is relatively higher than that of the proprietary formula, cerelac (1.82 g). Reported ash contents in complementary foods by other workers that included crayfish in their respective formulations were similar to the results obtained in this study (Abbey and Nkanga, 1998; Solomon, 2005).

The low moisture content of the samples (4.31-5.41 g/100 g) could be attributed to the hydrolysis of starch and protein macromolecules during seed sprouting that resulted in loss of water holding capacity.

Table 4. Anti-nutrient contents of maize-soyabean complementary foods (mg/100 g)

Anti-nutrient	Blend 1	Blend 2	Blend 3
Tannin	0.10^{a}±0.02	0.12^{b}±0.05	0.14^{c}±0.01
Phytate	0.15^{a}±0.01	0.16^{b}±0.02	0.18^{b}±0.02
Oxalate	0.02^{a}±0.02	0.02^{a}±0.01	0.03^{b}±0.02

Values are means ± SD of triplicate determinations. Means with the same superscript along each row are not significantly different (p<0.05).
Source: Authors

Mineral and vitamin A contents of maize-soyabean complementary food blends

Table 2 shows the minerals (calcium, iron, zinc) and vitamin A contents of maize-soyabean blends.

The fortified blends calcium content ranged from 560.45 to 620.12 mg/100 g. The increase over the unfortified sample was 34.29 to 48.58% and could be derived probably from high calcium content in bonga fish, crayfish and carrot. The proposed daily ration size of 65 g would provide 271 to 403 mg of calcium from the formulated complementary foods for infants 6-8, and children 9-11 and 12-23 months (Table 3). This amount is inadequate considering the World Health Organization recommendation of 525 g for ages 6-8, and 9-11 and 350 g for age 12-23 months, respectively (WHO, 1998). However, increasing the frequency of CFs meal consumption to 2 times per day would meet 72% of calcium needed from complementary foods.

The iron content of the fortified samples varies from 12.43 to14.25 mg/100 g. The significant (p< 0.05) increase in iron content after fortification varied from 32.31 to 60.10%. This could be as a result of addition of bonga fish and crayfish which are known sources of iron and enhancer of its bioavailability in the formulation. The 65 g proposed daily ration size would provide 5.94 to 8.07 mg of iron from complementary foods for infants 6-8 and children 9-11 and 12-23 months old (Table 3). This range is inadequate considering phytate restrictive effect on iron bioavailability. To overcome the constraint, administration of the ration size two times per day to infants would help meet the 97% of iron needed from CFs for 9-11 age groups and prevent iron deficiency (Dewey, 2013). Fewtrell et al. (2017) recommended 11 g/day for infants 6-11 months and 6 g/day iron for children, 12-23 months, respectively.

The fortified samples zinc content ranged from 8.27 to 10.67 mg/100 g. The observed increase follows same pattern as iron. The increase when compared with the unfortified blend varied from 2.52 to 4.47 g/100 g and could be attributed to the contributions from bonga fish and crayfish. The earlier proposed 65 g daily ration size would provide 2.66 to 5.14 mg of zinc from the formulated complementary foods for infants 6-8 and children 9-11 and 12-23 months (Table 3). Masters et al. (2017) reported that 4 to 5 mg of zinc should be provided by the daily ration of a fortified complementary food. Also, a recommendation of 4.1 mg zinc for ages 6-8 and 9-11 months, and 1.1 mg for age 12-23 months was made by the Joint FAO/WHO Expert Consultation (2002). The complementary food (blend 3) that provides 5.14 g zinc could be adequate to meet the zinc RDA for the age groups and 86% of zinc needed from complementary foods (Dewey, 2013). Breast milk intake partially covered calcium requirements; however, most iron and zinc RNIs need to be obtained from complementary foods (Ortenzi and Beal, 2021).

The vitamin A content of the samples ranged from 387.67 to 710.52 µg RE/100 g. Results show that fortified samples had values ranging from 162.46 to 322.85 µg RE/100 g and were significantly higher (p<0.05) than the unfortified sample and may be due to the retinol content in bonga fish and crayfish and high carotenoids contents as reported in carrots (Simon and Wolf, 1987). Noccolo et al. (2003) reported that carotenoids main physiological function is as precursor of vitamin A. The proposed daily ration size of 65 g would provide 251 to 461 µg RE/100 g of Vitamin A from the formulated complementary food blends for infants 6 to 8 and children 9 to 11 and 12 to 23 months old (Table 3). The Joint FAO/WHO Expert Consultation (2002) recommended 400 µg RE/100 g vitamin A Daily Intake for both age groups. However, 350 and 400 µg RE/100 g daily vitamin A intake were variously recommended by World Health Organization (WHO, 1998) for infants and children 6-11 and 12-23 months old. According to Ortenzi and Beal (2021), breast milk provides largely vitamin A requirements, with only 20% needed from complementary foods.

Anti-nutrientional contents of maize-soyabean complementary blends

The levels of anti-nutrient properties of CFs produced from dehulled, germinated and cooked maize-soybean are shown in Table 4. The tannin, phytate and oxalate contents range from 0.10-0.14, 0.15-0.18 and 0.02-0.03 mg/100 g for blends 1, 2 and 3, respectively. The results showed that the anti-nutrient contents were generally low due to the processing methods employed. The levels however increased by the increased addition of carrots in fortificants. The increase in tannin, phytic and oxalate contents of fortified samples was 20-40, 6-20 and 0-50% when compared with the unfortified sample and was significant (p<0.05). Soaking, cooking and germination have been reported by many investigators to cause reduction in phytic acid content in millets and chickpeas (Sarita and Singh, 2016; Shi et al., 2018). The decrease in phytic acid content by the pre-treatment processes may be due to phytase action and leaching of this compound in water (Sinha and Khare, 2017). Handa et al. (2017) also reported that germination removed heat

stable compounds in cereals and legumes such as tannins and hydrates. The observed values of tannin, phytate and oxalate were safe and could not result in adverse physiological effects when consumed since they were lower than the safe levels of 2.0, 5.0 and 2.2 g/100 g, respectively (Monri and Bassir, 1969).

Conclusion

Home-made complementary foods in sub-Sahara Africa are mostly produced from cereals and legumes and are frequently part of family foods such as porridges and gruels. The diets therefore have high energy content and consequently very low in nutrient density of micro-nutrients, such as iron, calcium and zinc, recorgnized as “problem nutrients” by World Health Organization (WHO). The complementary food formulated during this investigation using dehulled germinated maize-soybean flours fortified with *bonga* fish, crayfish, and carrot had shown to contain higher levels of protein, calcium, iron, zinc compared to cerelac the commercial complementary food. Therefore, household level production of the fortified complementary foods is recommended. This is feasible since the crops employed in the formulation are produced in large quantity in Nigeria. Also, the technologies which are easily accessible to both the rural and urban poor with less expensive equipment are evidently available at household level. When this is done, infants would be fed diets that will promote good health and prevent micronutrient deficiency diseases and protein energy malnutrition. Blend comprising 50% maize, 30% soyabean and 20% fortificant flours which contained the highest macro and micronutrients when compared with the others is considered the best in terms of nutritional content.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Analysis of different extraction solvents: Influence on some properties of aerial yam (*Dioscorea bulbifera*) starch

Ojo Mofoluwaso Olufunmilola[1*], Oni Kunle[2], Fortune Abidemi Femi[1], Idowu-Adebayo F[2,3], Balogun Adenike[4] , Ogunbande Babatunde Joseph[5] and Ude Margaret[6]

[1]Department of Food Science and Technology, School of Agriculture and Agricultural Technology, Federal University of Technology Minna, Niger State, Nigeria.
[2]Department of Food Science and Technology, Faculty of Agriculture, Federal University of Oye Ekiti, Ekiti State, Nigeria.
[3]Department of Agrotechnology and Food Sciences, Food Quality and Design Group, Wageningen University, the Netherlands.
[4]Department of Food Science and Technology, College of Food Technology and Human Ecology, Federal University of Agriculture, Makurdi, Benue State, Nigeria.
[5]Department of Food Science and Technology, College of Food Technology and Human Ecology, University of Agriculture, Abeokuta, Ogun State, Nigeria.
[6]Department of Food Science and Technology, College of Food Science and Technology, University of Mkar, Mkar, Benue State, Nigeria.

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Starch was extracted from aerial yam (*Dioscorea bulbifera*) using water, sodium hydroxide, ammonium oxalate, and oxalic acid as extraction solvents, oven-dried at 45°C for 24 h, and milled to flour. The starch obtained was evaluated for yield, chemical and functional properties using standard procedures. The sensory attributes of the starch cooked pastes were also determined. Aerial yam extracted with oxalate gave the highest yield of 20.20% and high amylose content was observed in the water extracted aerial yam samples. A higher purity of 90.45% was observed in the aerial yam starches extracted with oxalate. Water extracted aerial yam starch had the highest swelling power and solubility index of 48.44% and 25.75 g/g with the least gelation capacity of 4% with high pasting viscosities and also low pasting temperature and time. The cooked paste of the starch extracted with oxalic acid was the most acceptable in terms of appearance (8.60) and overall acceptability (8.16). The starch extracted with water had higher swelling power and a solubility index than other starches extracted. Extraction of aerial yam starch with different solvents resulted in variations in the characteristics of the starch, which can find wide applications in both food and non-food industries.

Key words: Aerial yam starch, extraction solvents, yield, chemical, functional, sensory properties.

INTRODUCTION

The carbohydrate cache is starch, which is one of the most important and necessary carbohydrates in the

*Corresponding author. E-mail: ojo@futminna.edu.ng. Tel: +2348065747751.

human diet. Starch is commonly obtained from sources like grains, legumes, and tubers, but it may also be derived from different portions of plants, including fruits, leaves, seeds, and roots (Karmakar et al*.,* 2014; Malumba et al*.,* 2017). Starch is a versatile commodity with numerous non-food and food applications. Approximately 70% of all starch used globally is used in the food business, with typical starches like corn, wheat, and potatoes serving as the primary sources in the majority of countries (Malumba et al*.,* 2017; Sanful and Engmann, 2016; Mahmood et al., 2017). Starch is a low-cost material that is used as a gelling agent, thickener, emulsion stabilizer, and water binding agent (Sanful and Engmann, 2016; Mahmood et al., 2017). Starch is a crucial raw ingredient for the food industry, but it is also widely used in the paper, chemical, textile, pharmaceutical, and biotechnological industries (Jiang et al*.,* 2020).

In Nigeria, yam (*Dioscorea* species) is the fifth-most-harvested crop. As a result, cultivation is primarily focused on these types. The primary carbohydrate in yam tubers is starch, which can make up to 80% of the dry mass (Zhu, 2015). The use of yam starches for various food and industrial purposes is receiving more attention (Otegbayo et al*.,* 2014). The aerial yam (*Dioscorea bulbifera*), sometimes known as potato yam or cheeky, is one of the marginalized, underutilized, and less-cultivated yam species but is recently attracting interest in food applications (Zhu, 2015; Ojinnaka et al*.,* 2016). According to Ojinnaka et al*.* (2016), it is being grown in West Africa, South East Asia, and South and Central America. In addition, it is being grown in Asia and Africa. *D. bulbifera* tuber contains a significant amount of amylose and amylopectin as well as a rich source of starch that serves as an essential dietary supplement. In order to address the growing need for starch with high functionality and with a growing population, the current focus is on finding options for producing starch with improved physicochemical and functional qualities (Makroo et al*.,* 2021). Starch must now be obtained from less well-known and underutilized sources due to expanding demand, rising costs from traditional sources, and starch's essential role in both the food and non-food industries. The use of starches from unusual sources, such as aerial yam, for commercial applications may provide a substitute for labor-intensively produced conventional starches, as well as cost savings in industries, as has been suggested for underutilized crops (Makroo et al*.*, 2021). There is therefore a need to explore the starch properties as the physicochemical properties of starch differ with its source, starch purity, and isolation with the practiced extraction procedures or processes, which involve three successive phases viz., anatomic fragmentation, cell breakage, and finally separation/purification processes (Liu, 2005). In general, the alkali and acid isolation methods are typically the two techniques that have been employed most frequently in the food sector, with various adjustments over time (Cardoso et al., 2006; Wang and Wang, 2001).

Researchers are exploring alternative sources for the extraction of starch due to the increased demand for this polysaccharide and the expanding range of its possible applications (Betancur-Ancona et al*.*, 2001). An unconventional source of starch derived from aerial yam starch may be viable at the industrial level; therefore, its properties must be thoroughly researched in order to have concrete viability and diverse application in industries. This may also be one of the recently developed, non-conventional starches that add value to the neglected sources. The physicochemical properties of starch differ with its source (Guinesi et al., 2006) and with the commonly extracted extraction procedures or processes. Successive characterization of starch depends on its purity and isolation. The type of starch selected for specific industrial purposes is selected based on its availability and physicochemical properties; these are influenced by the source from which the starch is extracted (Tao et al., 2019).

The rise in the population has stimulated a corresponding growing demand for starch with high functionality. Because of rising demand, rising costs from traditional starch sources, and the critical role of starch in the food and non-food industries, starch must now be sourced from previously unknown and underutilized sources. The trend toward adding value to underutilized food crops, such as *D. bulbifera* starch, is essential because it has a lot of potential in terms of the nutritional and functional qualities that might be used to create a variety of industrial products. The starch could be investigated for food applications as higher demands for conventional starch sources like cassava and corn increase. According to Sanful and Engmann (2016), the underutilization of aerial yam may be caused by a lack of available studies pointing out potential applications. Therefore, this study will provide information on yield, composition, and other quality characteristics of the aerial yam starch with the ultimate aim of promoting its usage, production, and suggesting plausible products that it could be incorporated into for the creation of new products and varieties.

MATERIALS AND METHODS

Materials procurement, preparation and handling

Aerial yam (*D. bulbifera*) bulbils were purchased from a farm in Gboko Local Government of Benue State and species identified at the College of Agriculture Yandev (AOCAY) Gboko, Benue State.

Extraction of aerial yam starch

The wet extraction method described by Ojinnaka et al. (2016) was adopted with slight modification for the aerial yam starch extraction (Figure 1). One kilogram of aerial yam bulbils was used for each of the extraction solvents. Aerial yam bulbils were sorted and washed to remove extraneous particles and adhering soil, and the skin was

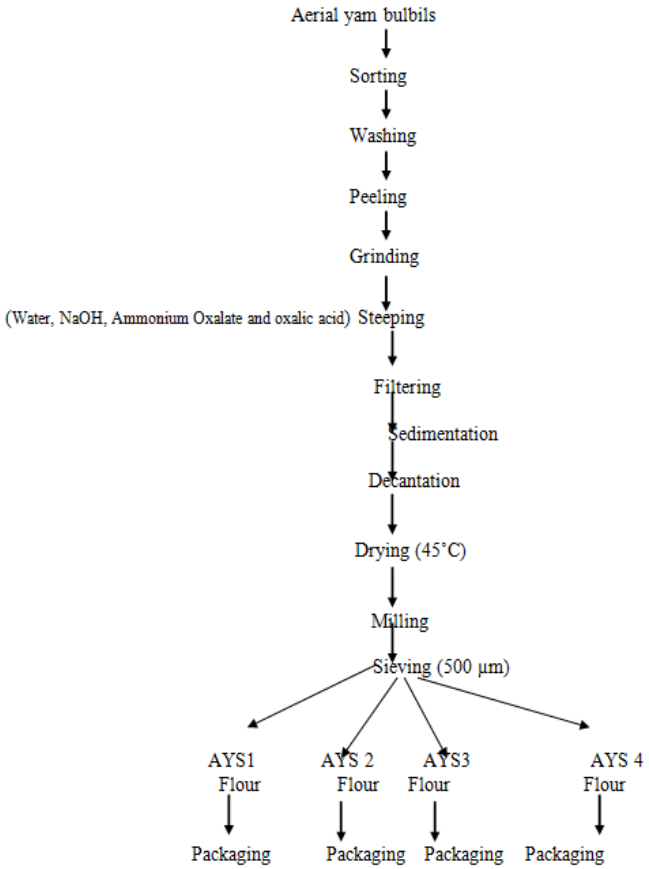

Figure 1. Aerial yam starch extraction using different solvents. AYS1 = Aerial yam starch extracted with water, AYS2 = Aerial yam starch extracted with sodium hydroxide (NaOH), AYS3 = Aerial yam starch extracted with ammonium oxalates, AYS4 = Aerial yam starch extracted with oxalic acid.
Source: Ojinnaka et al. (2016) modified.

peeled. The peeled aerial yam was ground using an attrition mill to a pulp with a ratio of 1:2 (aerial yam: water) for all the samples. The pulp was collected and steeped in the different extraction solvents (0.05 NaOH, ammonium oxalate, and 0.025 oxalic acids were prepared for the extraction and 0.25) for 5 h. The pH of the chemically extracted slurries was adjusted to 6.4, which corresponds to the pH of the control (water extracted starch). This also ensures that the starch fraction is unaltered at the extremes of pH. Then, the slurry was filtered through a sieve of 500 µm to separate the filtrate from the residue. The residue was allowed to sediment for 2 h, after which the supernatant liquid was decanted. The sedimentation and decanting processes were repeated twice to ensure that starch was not lost in the extraction solvents. The starch was then dried in an air convection oven (PEK medical) at 45°C for 24 h. This was followed by milling in an attrition mill (Model R175A) and weighing to obtain the various flours. The flour samples were packaged in polyethylene bags before further analysis.

Yield of extracted aerial yam starch

The amount of extracted starch from 1 kg of weighted aerial yam bulbils was used to calculate the starch yield.

$$\text{Yield capacity (\%)} = \frac{DW}{Weigh\ of\ edible\ portions} \times 100 \quad (1)$$

Where DW = Dry weight of starch recovered from the extraction.

Aerial yam starch analysis

Chemical analysis

Determination of proximate composition:

The method described by AACC (2003) was adopted for the proximate composition analysis and the degree of purity was calculated from the difference between 100 and the percentage of moisture, crude protein, lipids, fibre and ash content using the following equation:

% Purity = 100- (% moisture content + % crude protein + % lipids + % ash + crude fibre) (2)

Determination of amylose and amylopectin in starch

The amylose content of the extracted aerial starch samples was determined using the iodine colorimetric method reported by Zakpaa et al. (2010). A standard curve for amylose was prepared using different concentrations ranging from 0 to 70 mg of pure amylase. These were weighed into separate 100 ml volumetric flasks and 1 ml of ethanol, 10 ml of distilled water, and 2 ml of 10% sodium hydroxide were added. This was then heated in the water bath until a clear solution was obtained. The solution was cooled and diluted to the 100 ml mark with distilled water. Five millilitres of the solution was measured into a 500 ml volumetric flask and 100 ml of distilled water and acidified with drops of 6 M HCl and mixed thoroughly, followed by the addition of 5 ml of iodine solution, and this was made up to the 100 ml mark with distilled water. A spectrophotometer (UV - visible model 754, China) at a wavelength of 640 nm was used in measuring the absorbance of each. Then 100 mg of the aerial starch was weighed into a volumetric flask and the aforementioned procedures were repeated. The concentration of amylose was calculated from the standard curve. The percentage of amylopectin was calculated from the amylose obtained:

% Amylose = %Amylose of standard × Absorbance of sample Absorbance of standard (3)

While amylopectin was calculated as:

Amylopectin (%) =100 − Amylose (%) (4)

Determination of functional properties of aerial starch

The swelling power (SP) was determined using the method reported by Asogbon and Akintayo (2012). While the method described by Onwuka (2005) was adopted for the determination of solubility index (SI) and least gelation capacity (LGC).

Pasting properties determination

The pasting properties of the various aerial yam starches were determined using the Rapid Visco Analyzer (RVA, Perten Instrument) as described by Newport Scientific (1998) with slight modification. A starch sample of 3.5 g was weighed into the RVA canister. Twenty-five millilitres of distilled water was added and

thoroughly mixed into the slurry in the RVA can. The canister containing the starch slurry was then fitted into the RVA and the slurry was held at 50 to 95°C before being cooled back to 50°C for 12 min while being continuously stirred with a plastic paddle revolving at a speed of 160 rpm. The parameters evaluated were peak viscosity, setback viscosity, final viscosity, trough viscosity, pasting temperature, and time to reach peak viscosity.

Cooked starch paste preparation

Fifty grams of aerial yam starch were dissolved in 20 ml of water to form a water-starch mixture and heated for 10 min to acquire a cooked starch gel (Ojinnaka et al., 2016).

Sensory properties

The sensory properties of the cooked aerial yam starches were evaluated using the method described by Ihekoronye and Ngoddy (1985). Thirty (30) untrained panelists of the staff and students from the Department of Food Science and Technology University of Mkar, Mkar, Nigeria were recruited for the sensory evaluation using a 9 point Hedonic scale (1 = Extremely disliked to 9 = Extremely liked). Sensory parameters measured were appearance, aroma, mouthfeel, and general acceptability of the cooked aerial yam starch gel.

Statistical analysis

All analysis in this work was carried out in triplicates. Data were analyzed using the analysis of variance (ANOVA), while the means were separated by Duncan Multiple Range Test (DMRT), using the Statistical Package for Service Solutions (SPSS) Version 20.

RESULTS AND DISCUSSION

Yield and chemical properties of the various extracted starches

The result of the yield and chemical properties of the various extracted starches is presented in Table 1. The starch yield was calculated as the percentage of starch recovered. The yield of the starches ranged from 15.09 to 20.20% with AYS4 having the highest yield. Starch extraction using NaOH (AY2) had the lowest yield. Low extraction yield in NaOH may be explained mainly by loss of starch into the aqueous phase, which may be attributed to the presence of the alkali nature of the solvent and the existence of viscous water-soluble non-starch polysaccharides that impede the filtering process, thereby reducing the rate of sedimentation and carrying of the starch granules over into the wastewater (Ojinnaka et al., 2016). When the grated aerial yam was steeped into aqueous oxalic acid solutions, it was easier to separate the starch slurry for residual mass, probably because of the reduced viscosity which speeds up the settling rate, hence a high starch yield of 20.20% in oxalic acid. The yield values in this study fall within the range of 10.86 to 17.57% of the yield reported by Azima et al. (2020), although a slightly higher yield value for AYS4 is observed. A higher yield ranging from 42.59 to 62.92% was reported by Daiuto et al. (2005) for different solvent extractions of yam starch and 26.61 to 41.73% by Rugchati and Thanacharoenchanapas (2010) for yam tuber starch in Thailand.

All starch flours had moisture content ranging from 8.44 to 8.72%, with AYS1 having the lowest. The moisture content values were <10%. This supports starch stability at room temperature, shelf stability, and extended safe storage (Tortoe et al., 2019; Hayma, 2003). The crude protein content and ash of the samples varied significantly between 0.61 and 0.89% and 0.24 to 0.38%, respectively. The main factor influencing the extraction of starch has been identified as starch granule-associated protein. Proteins may be difficult to remove because they adhere to the surface of starch (Baldwin, 2001). The crude fat and fibre were only detected in the water extracted starch (AYS1). All the starch samples had high percentage purity values in the range of 90.08 to 90.45%, with AYS1 having the lowest percentage purity value. High purity indicates high starch content and signifies the quality of the starch product and also the efficiency of the solvent used in removing other polysaccharides, e.g. cell wall polysaccharides, proteins, and inorganic salts. This is important because the presence of other components could interfere with the functional properties of starch (Tapia et al., 2012). This could also be used to determine the efficiency of the solvents used with respect to aerial yam starch extraction. The extraction solvent had a significant impact on the amylose:amylopectin ratio, which was 23.03:66.05% (AYS1), 20.69:69.47% (AYS2), 20.03:70.42% (AYS3), and 20.10:70.29% (AYS4). There was a significant ($p< 0.05$) difference among the starch samples. The amylose content falls within the range of 17 to 33% and 18 to 29% for tapioca and potato, as reported by Vamadevan and Bertfort (2014). Starch functional properties such as swelling are mainly determined by two main components; these are the amylose and amylopectin ratio (Azima et al., 2016). Amylose content has been reported to influence starch resistance to digestion; it is less digestible than amylopectin (Faulks, 2003). Therefore, higher digestibility may be obtainable in AYS2, AYS3, and AYS4. The low amylose content of the samples indicates that when these starches are incorporated into food products, the swelling of starch will be enhanced (Addy et al., 2014). Since amylose tends to retrograde when foods are frozen and thawed, food with high amylopectin content will be useful in the preparation of foods for freeze-thaw processes (Woo et al., 2021).

Functional properties of aerial yam starches extracted

The results obtained for swelling power, solubility index, and gelation concentration of aerial yam starches extracted are shown in Table 2. The swelling power and the solubility index of the starches ranged from 36.98

Table 1. Effect of extraction solvents on yield and chemical composition of aerial yam starches.

Parameter (%)	AYS1	AYS2	AYS3	AYS4
Yield	$17.39^{b} \pm 1.15$	$15.09^{d} \pm 0.55$	$16.01^{c} \pm 0.96$	$20.20^{a} \pm 0.03$
Moisture content	$8.44^{c} \pm 0.02$	$8.71^{a} \pm 0.01$	$8.72^{a} \pm 0.01$	$8.55^{b} \pm 0.04$
Crude protein	$0.73^{b} \pm 0.01$	$0.89^{a} \pm 0.01$	$0.64^{d} \pm 0.00$	$0.67^{c} \pm 0.01$
Ash	$0.38^{a} \pm 0.02$	$0.24^{c} \pm 0.00$	$0.25^{c} \pm 0.02$	$0.30^{b} \pm 0.05$
Fat	0.25 ± 0.32	ND	ND	ND
Crude fibre	0.12 ± 0.00	ND	ND	ND
Purity	90.08	90.16	90.39	90.45
Amylose	23.03 ± 0.25	20.69 ± 1.02	20.10 ± 0.50	20.03 ± 0.78
Amylopectin	66.05 ± 0.25	69.47 ± 1.02	70.29 ± 0.50	70.42 ± 0.78

Values are means ± standard deviation of triplicate determinations. Values in the same row with different superscripts are significantly different ($p<0.05$). AYS1 = Aerial yam starch extracted with water; AYS2 = Aerial yam starch extracted with sodium hydroxide (NaOH); AYS3 = Aerial yam starch extracted with amonium oxalates; AYS4 = Aerial yam starch extracted with oxalic acid.
Source: Authors

Table 2. Effect of extraction of solvents on some functional properties of aerial yam starches.

Starch sample	SP (%)	SI (g/g)	LGC (%)			
			2	4	6	8
AYS1	$48.44^{a} \pm 1.50$	$25.75^{a} \pm 0.35$	-	+	+	+
AYS2	$44.23^{c} \pm 0.82$	$24.73^{b} \pm 0.52$	-	±	+	+
AYS3	$45.70^{b} \pm 0.50$	$25.40^{a} \pm 0.10$	-	±	+	+
AYS4	$36.98^{d} \pm 0.73$	$19.82^{c} \pm 0.01$	-	-	±	+

Values are Means ± standard deviation of triplicate determinations. Values in the same column with different superscripts are significantly different ($p<0.05$). **+ =** gel formed**,** ± = gel slightly formed and - = No gel formed. AYS1 = Aerial yam starch extracted with water; AYS2 = Aerial yam starch extracted with sodium hydroxide; AYS3 = Aerial yam starch extracted withamonium oxalates; AYS4 = Aerial yam starch extracted with oxalic acid SI= Swelling power; WAC = Water absorption capacity; LGC= Least gelation concentration.
Source: Authors

(AYS4) to 48.44% (AYS1) and 19.82 (AYS4) to 25.75 g/g (AYS3), respectively. There was a significant ($p<0.05$) difference in all the starch samples. High swelling capacity has been reported as part of the criteria for a good quality product (Princewill-Ogbonna and Ezembaukwu, 2015). Swelling power and solubility provide evidence of the magnitude of the interaction between starch chains within the amorphous and crystalline domains. The swelling power of starch depends on the water-binding capacity of starch molecules by hydrogen bonding (Lee and Osman, 1991). Srichuwong et al. (2005) stated that solubility could be attributed to the amount of amylose leaching out as the starch swells, the higher the solubility and the water uptake ability of the starches or the degree of dispersion of granules after cooking. The variations could be attributed to the differences in amylose content and extraction solvents.

Starch gelation is as a result of the re-association of hydrated and dispersed starch molecules. There were variations in the gelation concentration of the various starches. According to Otegbayo et al. (2014), starch's ability to form gel increases as LGC decreases. Firm gels were observed between 4 and 8%. AYS1 had the highest gelation capacity among the starches extracted and AYS4 recorded the lowest. Low gelation capacity starches (4-8%) will easily form gel, implying that they can find wide applications in industries where gelation is highly desired, such as pharmaceuticals and paper, as well as in food industries as thickeners, stabilizers, or gelling agents. They can also be used industrially with slight or no modification (Wang and Wang, 2001; Lawal, 2004). The LGC result compares favourably with starches extracted from different yam (*D. bulbifera*) species in Nigeria, which ranged from 2 to 10% (Otegbayo et al., 2014) and cassava starch (8%) reported by Ojo et al. (2017).

Effect of extraction solvent on the pasting properties of aerial yam starches

The pasting properties of aerial yam starches extracted with different solvents are shown in Table 3. Pasting

Table 3. Effect of extraction solvents on the pasting properties of aerial yam starch.

Sample	Pasting parameters (RVU)					P_{temp} (0°C)	P_{time} (min)
	PV	TV	BV	FV	SV		
AYS 1	$302.17^{a} \pm 0.06$	$152.40^{c} \pm 0.00$	149.73a ± 0.06	$381.20^{a} \pm 0.00$	$228.80^{a} \pm 0.00$	$77.20^{c} \pm 0.10$	$5.23^{b} \pm 0.03$
AYS 2	$259.40^{c} \pm 0.06$	$139.80^{d} \pm 0.00$	119.60b ± 0.01	$328.33^{c} \pm 0.58$	$188.53^{c} \pm 0.58$	$81.50^{a} \pm 0.06$	$5.47^{a} \pm 0.02$
AYS 3	$255.37^{d} \pm 0.29$	$156.40^{b} \pm 0.01$	$98.96^{d} \pm 0.28$	$305.50^{d} \pm 0.00$	$149.10^{d} \pm 0.01$	$80.08^{b} \pm 0.00$	$5.33^{b} \pm 0.01$
AYS 4	$265.00^{b} \pm 0.30$	$160.47^{a} \pm 0.06$	$104.52^{c} \pm 0.33$	$356.80^{b} \pm 0.00$	$196.33^{b} \pm 0.06$	$80.10^{b} \pm 0.03$	$5.33^{b} \pm 0.01$

Values are Means ± standard deviation of triplicate determinations. Values in the same column with different. Superscripts are significantly different ($p<0.05$). PV= Peak viscosity, TV= Trough viscosity, BV= Breakdown viscosity, FV= Final viscosity, SV= Setback viscosity. Ptemp = Pasting temperature**,** Ptime= Pasting time, AYS1 = Aerial Yam Starch extracted with Water, AYS2 = Aerial Yam Starch extracted with NaOH, AYS3 = Aerial Yam Starch extracted with Ammonium oxalates, AYS4 = Aerial Yam Starch extracted with Oxalic acid.
Source: Authors

properties of starch are of importance as they influence the amount of energy required to process starch, the stability of the starch during processing as well as the stability of the products with its probable use in the food industry (Crosbie and Ross, 2007; Atwijukire et al., 2019). The pasting properties are also dependent on the rigidity of starch granules, which in turn affects the swelling potential of granules (Ritika et al., 2010). The peak viscosity and trough viscosity ranged from 255.37 (AYS3) to 302.17 RVU (AYS1) and 139.80 (AYS2) to 160.47 RVU (AYS4), respectively while the breakdown viscosity ranged from 98.96 (AYS3) to 149.73 RVU (AYS1). The peak viscosity indicates the highest viscosity attained by starch slurry during heating. The high peak viscosity values recorded for AYS1 starch are not surprising as high peak viscosities are associated with the degree of swelling of the starch granules during heating (Chandrasekara and Kumar, 2016). Peak viscosity may be associated with starch crystalline structure and amylopectin branch chain distribution. During gelatinization, the crystallinity of starch is lost and amylose leaches out of amylopectin, hence leading to an increase in viscosity. Trough viscosity is related to the degree of gelation of cooked starches and it also measures the ability of the paste to withstand breakdown during cooling (Cornejo-Ramirez et al., 2018). Breakdown viscosity measures the susceptibility or vulnerability of the cooked starch sample to disintegration. A high peak viscosity value is associated with a high breakdown viscosity. High breakdown value is desirable in products that are to be kept at high temperatures for a long time (Princewill-Ogbonna and Ezembaujkwu, 2015). The final viscosity of the various starches studied ranged from 305.50 RVU (AYS3) to 381.20 (AYS1) and setback viscosity ranged between 149.10 (AYS3) and 228.80 RVU (AYS1). Among the aerial yam starch samples, AYS1 had the highest setback value. This means that they are more predisposed to retrogradation. AYS1 could find more use in comparison with the AYS2, AYS3, and AYS4 starches in this study in food products such as noodles where high retrogradation is desired and products which undergo loss of viscosity and precipitation as a result of retrogradation for example soups and sauces. Other attributes closely associated with the pasting properties of starch are the peak temperature and time. The peak temperature ranged from 77.20 (AYS1) to 81.50°C (AYS2). The peak temperature is the lowest temperature at which maximum starch granule swelling is attained (Crosbie and Ross, 2007). High pasting temperature indicates high resistance to swelling. The low pasting temperature recorded for AYS1 implies that starch reached maximum swelling much earlier than the other solvents extracted starches. This is advantageous as it implies lower energy use when used in food applications. The results for pasting behaviour showed that higher amylose content was associated with a lower pasting temperature and a higher peak viscosity in these starches. The peak time, which is related to cooking time, ranged between 5.23 (AYS1) and 5.47 min (AYS2). Interestingly, the pasting time and temperature range are in agreement with those reported for a different cultivar of *D. bulbifera* by Otegbayo et al. (2014).

Sensory properties of aerial yam starches cooked paste

The results obtained for the sensory properties of aerial yam starch cooked paste are presented in Table 4. The appearance, mouthfeel, flavour and general acceptability of the cooked starch paste ranged from 5.75 (AYS1) to 8.60 (AYS4), 6.15 (AYS4) to 6.85 (AYS3), 6.50 (AYS4) to 6.95 (AYS3) and 7.10 (AYS3) to 8.16 (AYS4), respectively. There was a significant difference ($P \leq 0.05$) among the cooked starch pastes with respect to appearance and overall acceptability. The flavour and mouthfeel were not affected by the extraction solvents as there was no significant difference among the cooked pastes. Aerial yam starch extraction using oxalic acid was recorded as the highest in appearance and acceptability. Visually, the starch sample extracted using oxalic acid resulted in a striking white colour due to its bleaching

Table 4. Mean sensory scores of aerial yam starch cooked paste as influenced by extraction solvents

Sample	Appearance	Mouth feel	Flavour	Overall acceptability
AYS 1	$5.75^{cd} \pm 0.41$	$6.65^{ab} \pm 1.10$	$6.70^{a} \pm 0.15$	$6.20^{c} \pm 0.24$
AYS 2	$6.15^{c} \pm 0.30$	$6.25^{ab} \pm 0.90$	$6.75^{a} \pm 0.26$	$7.23^{b} \pm 0.40$
AYS 3	$7.85^{b} \pm 0.45$	$6.85^{a} \pm 0.25$	$6.95^{a} \pm 0.55$	$7.10^{b} \pm 0.60$
AYS 4	$8.60^{a} \pm 0.25$	$6.15^{ab} \pm 0.72$	$6.50^{a} \pm 1.03$	$8.16^{a} \pm 0.18$

Values are Means ± standard deviation of triplicate determinations. Values in the same column with different superscripts are significantly different ($p<0.05$). AYS1 = Aerial Yam Starch extracted with water, AYS2 = aerial yam starch extracted with NaOH, AYS3 = aerial yam starch extracted with ammonium oxalates, AYS4 = aerial yam starch extracted with oxalic acid.
Source: Authors

Plate 1. Visual presentation of aerial yam starch flours using different extraction solvents. AYS1 = Aerial yam starch extracted with water, AYS2 = Aerial yam starch extracted with sodium hydroxide (NaOH), AYS3 = Aerial yam starch extracted with amonium oxalates, AYS4 = Aerial yam starch extracted with oxalic acid.
Source: Authors

ability as a reducing agent, while the starch sample treated with water, sodium hydroxide and ammonium oxalate gave a less appealing sight (Plate 1) probably because of the less effect on enzymatic browning. These visual colour differences could restrict the use of the starch samples in products where the colour changes are not desired.

Conclusion

This study showed that aerial yam starches extracted using different solvents showed appreciable differences in their yield, chemical, and functional properties. Aerial yam starch extraction was considerably faster, with ammonium oxalate giving the highest yield of 18.01%. This could be more economical with functional properties, revealing the diverse applications in both food and non-food industries. The cooked paste from aerial yam starch extracted using oxalic acid as extraction solvent was the most acceptable.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Increased antibacterial activity of *Angelica koreana* and *Arnebia euchroma* extracts fermented by *Lactobacillus acidifarinae* against methicillin-resistant *Staphylococcus aureus*

Jae-Goo Kim[1], Yunji Cha[2], Seung-Bo Yang[3], Jiyoung Kim[2] and Ki-Young Kim[1*]

[1]Graduate School of Biotechnology, Kyung Hee University, Gyeonggi-do, Republic of Korea.
[2]Cosmogen Co., Ltd. Suwon-si, Gyeonggi-do, Republic of Korea.
[3]Department of Korean Internal Medicine, College of Korean Medicine, Gachon University, Seongnam-si, Gyeonggi-do, Republic of Korea.

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The increasing epidemic of methicillin-resistant *Staphylococcus aureus* (MRSA), one of the most important hospital and community pathogens, has led to a demand for new agents to treat the infection. Natural products may be used to reduce this problem with low side effects. The objective of this study was to determine the antibacterial effect of fermented extracts of *Angelica koreana* and *Arnebia euchroma* by *Lactobacillus* spp. against *S. aureus*, which were tested by disk diffusion test. Extracts of *A. koreana* and *A. euchroma* showed a clear zone of 15.5 ± 1.1 and 15.9 ± 2.2 mm, respectively. Fermented extracts by *Lactobacillus* sp. showed more improved antibacterial activity against *S. aureus* than the extracts.

Key words: MRSA; *Lactobacillus* spp.; fermentation; disk diffusion test; plant extract.

INTRODUCTION

Staphylococcus aureus is a major pathogen causing nosocomial infections. The emergence of antibiotic-resistant strains of *S. aureus* that caused infections among hospitalized patients is a severe problem worldwide (Li and Webster, 2018).

For example, the rate of hospital-acquired MRSA reached 50.4% in China and MRSA is causative of almost 44% of cases and over 20% of excess mortality among healthcare-acquired infections in Europe (Guo et al., 2020). Treatment options for MRSA infection are currently limited because most MRSA strains are resistant to widely used antibiotics such as lactams, macrolides, aminoglycosides, and fluoroquinolones (Schentag et al., 1998; Tacconelli et al., 2008; Kaur and Chate, 2015). Therefore, it is necessary to find alternative treatments to prevent and control MRSA infections.

Angelica koreana, also named *Ostericum koreanum*, has traditionally been used in oriental Korean medicine to

*Corresponding author. E-mail: kiyoung@khu.ac.kr.

treat the common cold, and to reduce rheumatic pains or headaches. This plant has been reported for various biological activities including anti-tumor, anti-microbial, antioxidant, and anti-inflammatory effects (Kang et al., 2009; Shin, 2005; Park et al., 2007, 2008).

Arnebia euchroma Royle is a well-known traditional herb used for various skin diseases in Iranian tribal medicine (Ashkani-Esfahani et al., 2012). Shikonin derivatives isolated from the roots of *A. euchroma* have been reported for antimicrobial, anti-inflammatory and anti-tumor activities and thus to be considered as important compounds for potential medicinal use (Kim et al., 2001).

The bioconversion process could enhance the biological activities of medicinal plants and herbs. A previous study showed that lactic fermented herbal teas have more composition in phenolic, flavonoid compounds (Ibrahim et al., 2014). Another study also showed that the antioxidant and antibacterial activities of medicinal plants fermented by fungi are increased compared to non-fermented control (Dong et al., 2015). *Lactobacillus*-fermented *Artemisia princeps* has been used as a functional component to increase the growth performance, meat lipid stability, and intestinal health of chickens (Kim et al., 2012).

This study aimed to evaluate the antibacterial effects of *A. koreana* and *A. euchroma* extracts(AK and AE, respectively) after fermentation by *Lactobacillus* spp. against *S. aureus* using the disk diffusion and biofilm formation method.

MATERIALS AND METHODS

Raw materials

The roots of *A. koreana* and *A. euchroma* Royle were commercially purchased (Barumhanyak, Korea). 1 kg each of dried plant roots was immersed in 5-10 volumes of 70-80% ethanol. They were extracted by maceration for overnight with constant shaking at 24 °C 3 times. The obtained extracts were filtered with Whatman 2 filter paper to discard impurities. The filtered extract was concentrated under reduced pressure in a rotary concentrator and dried to obtain a solid content of the extract.

Bacterial strains and culture

S. aureus (CCARM3505, MRSA and CCARM3506, QRSA), *Lactobacillus acidophilus* (KACC12419, AD), *Lactobacillus acidifarinae* (KACC16342, AF) and *Lactobacillus acidipiscis* (KCTC12394, AP) were purchased from CCARM (The Canadian Centre for Agri-Food Research in Health and Medicine) and KCTC (Korean Collection for Type Cultures). All strains were kept in 20% glycerol at -70°C. *S. aureus* was cultured in tryptic soy broth (TSB, BD Difco, Franklin Lakes, USA) containing tryptone 17 g, soytone 3 g, glucose 2.5 g, sodium chloride 5 g, and dipotassium phosphate 2.5 g at 37°C for 24 h. *Lactobacillus* spp. were cultured in MRS containing Lactobacilli MRS Broth 55 g/L (BD Difco, Franklin Lakes, USA) at 37 °C for 24 hours under anaerobic condition (Bae et al., 2019).

Fermentation of *A. koreana* and *A. euchroma* with *Lactobacillus* spp.

Extracts of *A. koreana* and *A. euchroma* were inoculated to be 1% of the total volume at 10^7 CFU/mL of *Lactobacillus* spp. Fermentation was carried out in 14 mL round tube for 24-96 h at 37°C under anaerobic condition. Filtration of the supernatant was done using a nominal 0.22 µm filter (ProLabs, Korea) to remove residual cells (Hashemi et al., 2017). It was repeated three or more times to produce fermentations.

Antimicrobial susceptibility testing

Bacterial suspensions with a turbidity equivalent to a McFarland standard of 0.5 were swabbed evenly onto TSA plates with a sterile cotton swab for the disk diffusion method. Antibiotic disks containing ampicillin, plant extracts, and fermented extracts were placed on TSA plates. The plates were incubated at 37 °C for 24 h and then the inhibition zone diameters, including the diameter of the disk, were measured (Dušková and Karpíšková, 2013). The test was repeated three or more times and all data are the average ± STDEV.

Biofilm formation assay

A slightly modified biofilm formation assay was used (Hobby et al., 2012; O'Toole, 2011). The 6-well plate was incubated with extracts at 37°C for 24 h; the wells were gently washed twice with 200 µL of phosphate-buffered saline (PBS) to remove all planktonic cells. After aspiration of planktonic cells, adherent biofilms were fixed with absolute ethanol before staining with 200 µL 0.2% crystal violet solution (Thermo Fisher Scientific, USA) for 5 min. Following aspiration of the stain, wells were washed three times with PBS and air-dried. A quantitative assessment of biofilm formation was then taken by adding 33% acetic acid and incubating for 10 min. The absorbance of eluate was calculated from optical density (OD_{570}) values measured using a microplate reader (BioTek Instruments, Korea). The assay was repeated three or more times and all data are the average ± STDEV.

Cell viability assay

A slightly modified MTT assay was used to test the cell viability of HaCaT cells (Park et al., 2021). Briefly, HaCaT cells in Dulbecco's modified eagle's medium (DMEM) at a density of 10^4 cells are cultured in a 96-well plate for 24 h. Serum-free medium containing extracts or fermented extracts was added to the wells. After 24 h of incubation, MTT (3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide, Sigma) was added, followed by incubating for 3 h at 37°C. The solution was then discarded, and cells were suspended in 100 µL of DMSO (Dimethyl Sulfoxide, Junsei, Japan). Absorbance was calculated from optical density (OD_{540}) values measured using a microplate reader (BioTek Instruments, Korea). The assay was repeated three or more times and all data are the average ± STDEV.

Ames mutagenicity assay

The Ames mutagenicity assays were performed, according to the method of manufacturer recommendation (Xenometrix, Switzerland) (Flückiger-Isler and Kamber, 2012). Both tester strains, *Salmonella Typhimurium* TA98 and *Escherichia coli* WP2 *uvrA*, were grown in growth medium overnight at 37°C. The test cultures were exposed to the indicated concentrations of extracts for 90 min in liquid

Table 1. Zone of inhibition by plant extracts against bacterial strains.

Strains	Materials	Clear zone (mm)
S. aureus (CCARM3505)	Ampicillin	26.0 ± 0
	A. koreana	15.5 ± 1.1
	A. euchroma	15.9 ± 2.2

Source: Authors

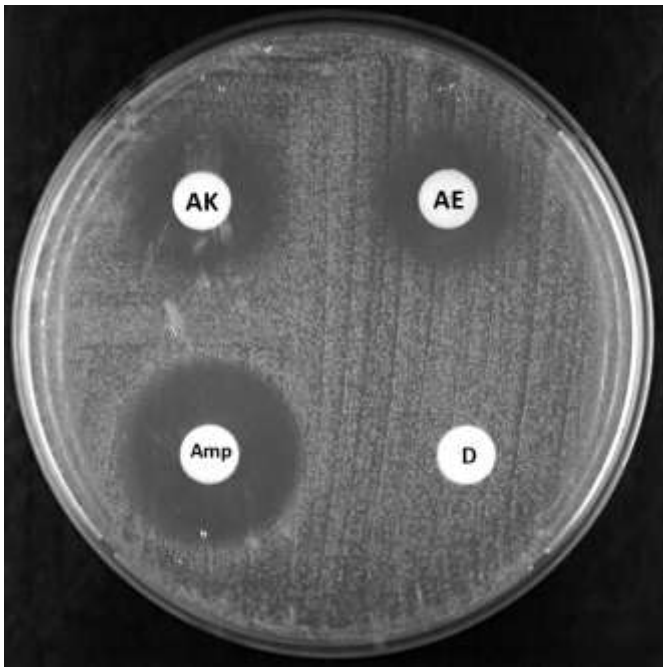

Figure 1. Antibacterial activity of *A. koreana* (AK) and *A. euchroma* (AE) extract against *S. aureus* (CCARM3505) with disk diffusion method. Bacteria with a McFarland standard of 0.5 were swabbed evenly onto plates. Disk containing 30 µL of ampicillin and extracts were placed onto the plates. D = DMSO; Amp = Ampicillin.
Source: Authors

minimal exposure media in a 24-well plate. After each well of the 24-well plates was added with 2.6 ml of indicator medium, 100 µL aliquots of culture were then dispensed into a 96-well plate. The 96-well plates were incubated at 37°C for 48 h. The number of positive (yellow) wells out of 48 wells per replicate was compared to the number of revertants from the negative control.

RESULTS AND DISCUSSION

Antibacterial test

The extracts of AK and AE showed clear zone sizes of 15.5 ± 1.1 and 15.9 ± 2.2 mm against *S. aureus*, respectively (Table 1 and Figure 1). Lactic acid bacteria (LAB), particularly those belonging to beneficial and non-pathogenic bacteria have traditionally been used in the food industry. Recent studies have shown their preventive effect against infection. For example, the incidence of infections and acute diarrhoea in children is reduced (Gleeson et al., 2011; Sur et al., 2011). In addition, LAB is widely used as functional foods, and LAB fermentation products and supernatant are also useful as cosmetic ingredients.

Streptococcus spp., *Lactobacillus* spp., and *Lactococcus* spp. are mainly applied for fermentation. Various substrates such as soybeans, fruit, and plants are used for culture (Izawa and Sone, 2014). With three *Lactobacillus* spp., AK and AE extracts were separately fermented for 72 h. Fermentation with AF showed a larger clear zone size of 19.2 ± 2.3 mm compared with non-fermented AK extract. In the case of AE extract, fermentation with AF also showed a prominent clear zone

Table 2. Zone of inhibition of 72 hours-fermented plant extracts against *S. aureus* (CCARM3505).

Extracts	Fermentation strains	Clear zone (mm)
		S. aureus
A. koreana	-	15.0 ± 0.9
	L. acidophilus (AD)	18.7 ± 3.2
	L. acidifarinae (AF)	19.2 ± 2.3
	L. acidipiscis (AP)	12.0 ± 0
A. euchroma	-	14.8 ± 1.1
	L. acidophilus (AD)	15.0 ± 2.1
	L. acidifarinae (AF)	16.6 ± 1.5
	L. acidipiscis (AP)	12.0 ± 0

Source: Authors

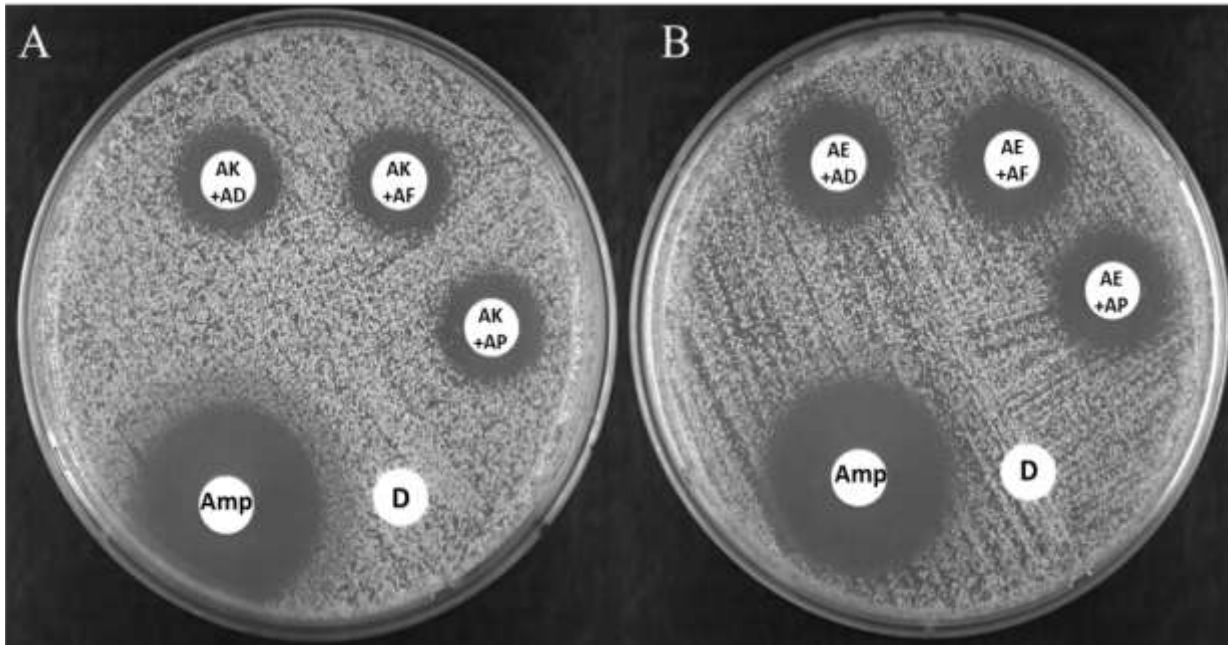

Figure 2. The antibacterial activities of 72 hours-fermented extract with *Lactobacillus* spp. against *S. aureus* (CCARM3505). (A) The extract of *A. koreana* (AK) fermented by *L. acidophilus* (AD), *L. acidifarinae* (AF), or *L. acidipiscis* (AP) for 72 h; (B) The extract of *A. euchroma* (AE) fermented by *L. acidophilus* (AD), *L. acidifarinae* (AF) or *L. acidipiscis* (AP) for 72 hours. D = DMSO; Amp = Ampicillin.
Source: Authors

size of 16.6 ± 1.5 mm (Table 2 and Figure 2). To test how long time of fermentation is most effective, AK and AE extracts were fermented with AF for indicated times. Both extracts showed clear zone sizes of 17.8 ± 2.6 and 16.4 ± 1.9 mm, respectively, when fermented for 72 hours (Table 3 and Figure 3).

Various combinations of the two fermented extracts were tested for synergistic effect. The largest clear zone size was expressed as 16.4 ± 2.8 mm by AK+AF: AE+AF=3:2 combinations (Table 4 and Figure 4). AK and AE extracts showed antibacterial activity against *S. aureus* but to make the extract showed higher activity, the fermentation by LAB was applied.

Fermentation with LAB is used in various fields, and in this study, three types of LAB were tested. Among the three LAB, the largest clear zone was observed in the fermented extract with AF, and the greatest clear zone was observed with 72 h fermentation, which was selected

Table 3. Zone of inhibition by fermented extracts for indicated time against *S. aureus* (CCARM3505).

Strains	Materials	Clear zone (mm) Fermentation time (h)			
		24	48	72	96
S. aureus (CCARM3505)	AK+AF*	15.6 ± 1.9	15.3 ± 1.8	17.8 ± 2.6	15.2 ± 0.3
	AE+AF*	15.6 ± 2.1	15.4 ± 2.5	16.4 ± 1.9	14.5 ± 0

Source: Authors

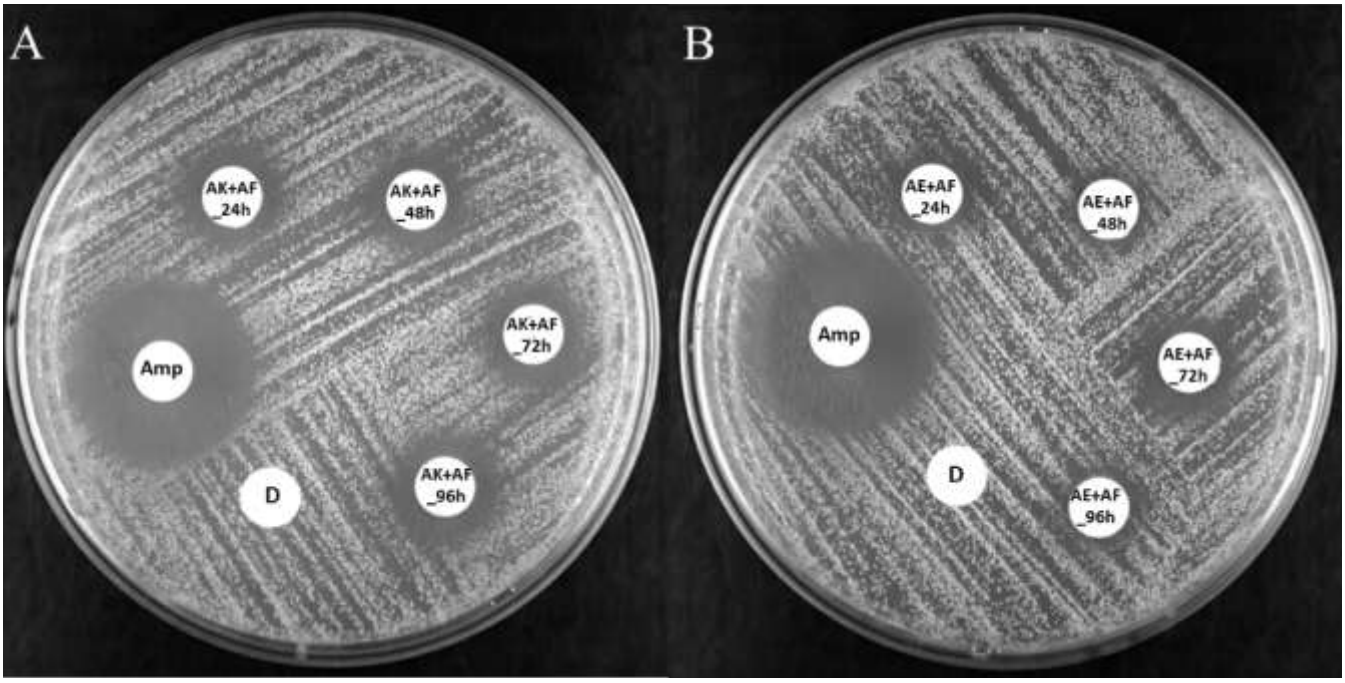

Figure 3. The antibacterial activities of fermented extract with *L. acidifarinae* against *S. aureus* (CCARM3505). (A) The antibacterial activities of AK fermented for indicated time (24, 48, 72 and 96 h); (B) The antibacterial activities of AE fermented for indicated time (24, 48, 72 and 96 h). D = DMSO; Amp = Ampicillin.
Source: Authors

Table 4. Zone of inhibition by combined treatment of fermented extracts against *S. aureus* (CCARM3505).

Ratio of *AK+AF:*AE+AF	Clear zone (mm) *S. aureus*
5:0	14.9 ± 2.4
4:1	16.1 ± 1.9
3:2	16.4 ± 2.8
2:3	14.2 ± 3.1
1:4	14.5 ± 1.8
0:5	15.5 ± 2.0

*AK+AF: *L. acidifarinae* (AF) fermented *A. koreana* extract, AE+AF: *L. acidifarinae* (AF) fermented *A. euchroma* extract.
Source: Authors

as the best fermentation condition. AF was first identified in 2005 (Vancanneyt et al., 2005), but research about AF has not been largely conducted. Therefore, the increased antibacterial activity by fermentation with AF found in this study appeared to be meaningful.

Biofilm formation inhibition

Surface adhesion of bacteria is an essential step and is necessary for bacteria to infect host in their environment. The role of biofilm is to attach to abiotic surfaces, epitheliums, and interfaces in multicellular organisms (Berne et al., 2015).

Biofilm serves to promote bacteria survival by preventing antibiotic activity and host immune responses,

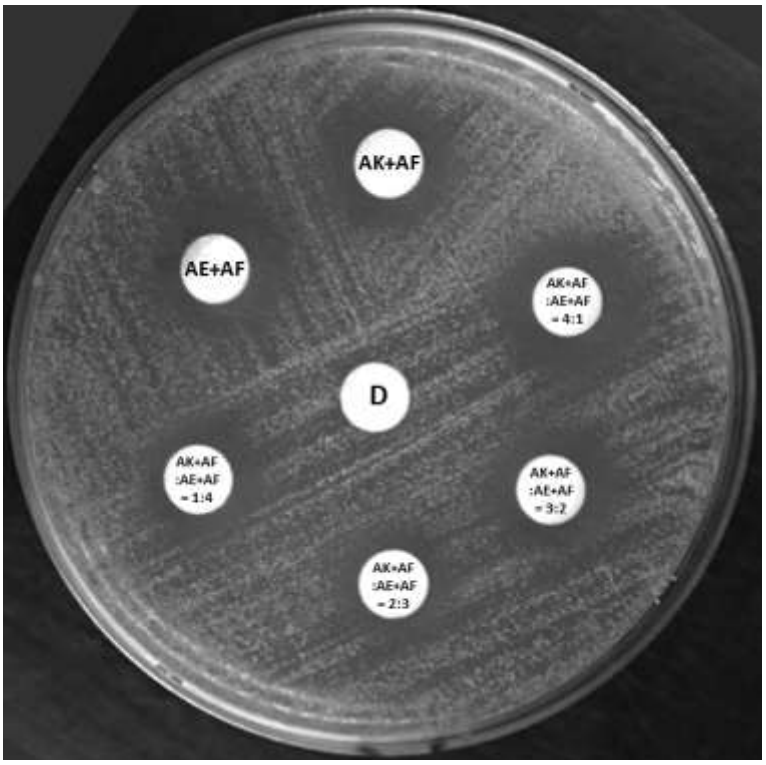

Figure 4. The antibacterial activity of combined treatment of fermented extracts against *S. aureus* (CCARM3505), D = DMSO; Amp = Ampicillin. Source: Authors

so it is considered as a good target for the biofilm forming pathogen (Schulze et al., 2021). To examine the potential anti-biofilm formation effect of the fermented extracts, *S. aureus* was cultured in the presence of the fermented extracts and biofilm formation was detected using crystal violet biofilm assay. Each fermented extract and combination of both fermented extracts dramatically decreased *S. aureus* biofilm formation by over 50%. The most prominent anti-biofilm formation activity was observed with AE extract fermented by AF. 100 µg/mL of AK and AE extracts fermented with AF inhibited biofilm formation by 44.19 ± 13.06 and 18.57 ± 4.81 compared with untreated bacteria, respectively. Combined treatment of both fermented extracts showed 35.55 ± 6.53% inhibition of *S. aureus* biofilm formation (Figure 5). These results suggest that AF-fermented AK and AE extracts are good candidates to control the biofilm forming pathogenic *S. aureus* (Ames et al., 1973).

Cell viability

Although AK extract, AE extract, and fermented extracts exhibit antibacterial effects against *S. aureus*, it is important to test toxicity with normal mammalian cell lines including HaCaT (Human keratinocyte cell line). AK extract showed cell viability lower than 20% at 250 µg/mL treatment (Figure 6A) but AK extract fermented with AF for 72 h showed reduced toxicity with higher than 80 and 60% of cell viability at 250 and 1000 µg/mL treatment, respectively (Figure 6C). AE extract and fermented AE extract did not influence the cell's viability (Figure 6B, 6D).

Ames test

The Ames test was repeated three times without S9 metabolic activation. Fermented extract and combined treatment did not show any mutagenic activity in TA98 and WP2 *uvrA* strains without metabolic activation (Table 5). None of the investigated fermentations showed any potential mutagenic effects.

Conclusion

This manuscript showed that *A. koreana* and *A. euchroma* extracts exhibited the antibacterial effect against *S. aureus*. Fermented extracts by *L. acidifarinae* showed improved antibacterial effect and anti-biofilm formation activity against *S. aureus* with reduced animal

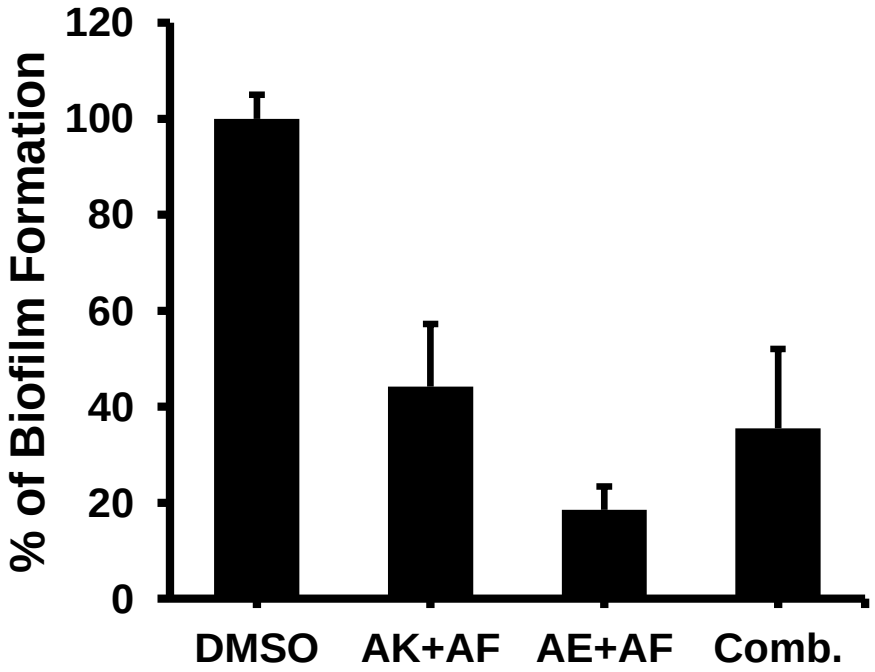

Figure 5. Fermented extract inhibited *S. aureus* biofilm formation. Effect of fermented extracts (100 µg/mL) against *S. aureus* biofilm formation was detected using crystal violet solution. AK+AF (72 h-fermented), AE+AF (72 h-fermented) and combined treatment of both fermented extracts (Comb., AK+AF: AE+AF = 3:2) were used.
Source: Authors

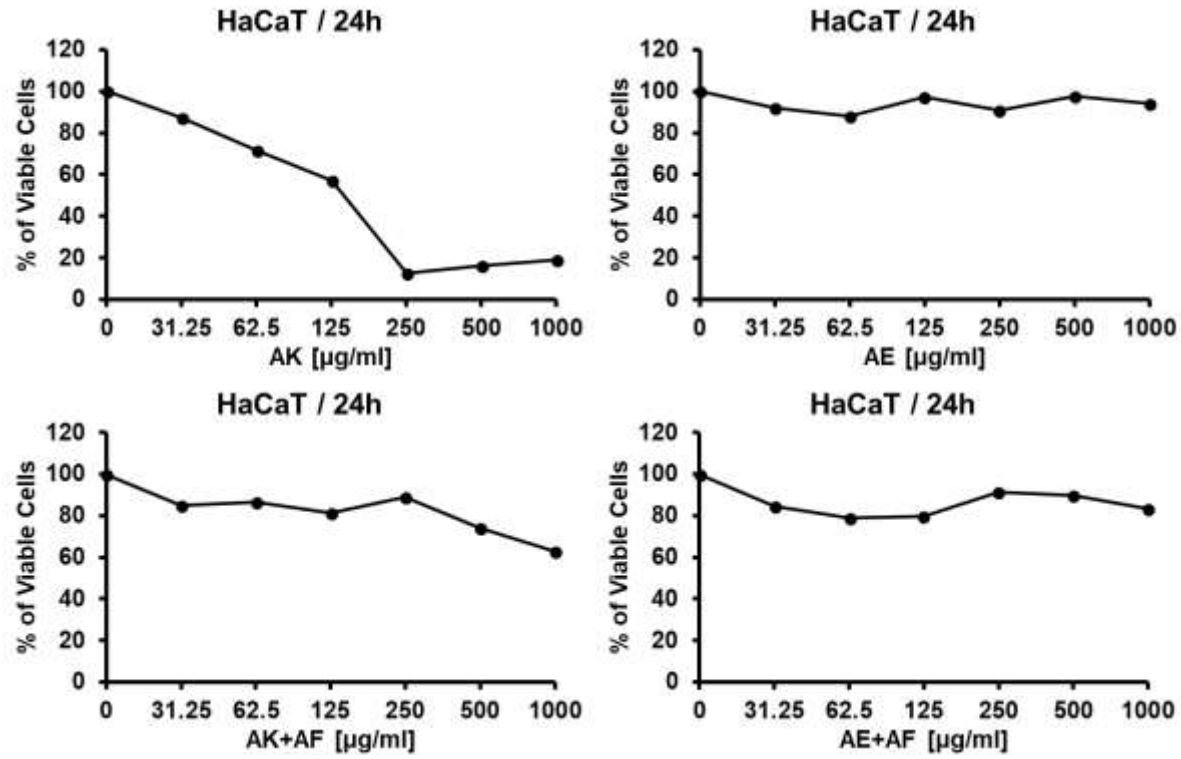

Figure 6. Fermented extracts reduced the toxicity of AK extracts. Effects of (A) AK, (B) AE, (C) AK+AF (72 h-fermented) and (D) AE+AF (72 h-fermented) on the growth of HaCaT cells were tested.
Source: Authors

Table 5. Mutagenicity of fermented materials toward *S. typhimurium* TA98 and *E. coli* WP2 *uvrA* strains without metabolic activation.

Materials	Dose (µg/mL)	Number of revertants (Mean)	
		***S. typhimurium* TA98**	***E. coli* WP2 *uvrA***
DMSO	-	4	8
*AK+AF	40	8	4
*AE+AF	40	36	4
AK+AF:AE+AF =3:2	80	16	8

*AK+AF: *L. acidifarinae* (AF) fermented *A. koreana* extract, AE+AF: *L. acidifarinae* (AF) fermented *A. euchroma* extract.
Source: Authors

cell toxicity compared with non-fermented extract.

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CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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