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"Lakh vzla" A Popular Fermented Beef Bone Base Flavour Enhancer from Mafa People in Northern Cameroon

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Authors' contributions

This work was carried out in collaboration between all authors. Author DDR designed the study, performed the statistical analysis, wrote the protocol, and wrote the first draft of the manuscript. Authors BJ and BD managed the laboratory analyses of the study. Authors ENJJ and EFX managed the literature searches, read and corrected the first draft of the article. All authors read and approved the final manuscript.

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ABSTRACT

"Lakh vzla" is one of the most interesting food flavour enhancer used for notabilities amongst Mafa people from Northern Cameroon. With the aims at valorizing this African food enhancer, we studied artisanal processing production and investigated the principal microbiological activities and biochemical modifications which occur during its processing. It was found that the main substrate for this fermentation was bones powder and the source of microorganisms the fat used as melting ingredient. The fermentation time was found to be five to seven days. The process that degrades

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bone during “Lakh vzla” production was found to be similar to that of diagenesis. The first step in the process involves elimination of the organic collagen fraction by the action of bacterial collagenases. This collagenase may mainly be produced by isolated Bacillus sp. strains. The collagenases break down protein into peptides which are subsequently reduced to their constituent amino acids involved in flavouring and particular odour of “Lakh vzla”. Improvement of the process are proposed to ameliorate both the process production and quality of the end product.

Keywords: Fermentation; African; food enhancer.

1. INTRODUCTION

Artisanal African food and beverage are gaining interest amongst scientist for their cultural values [1,2]. This interest is extended into food sectors for valorisation at industrial levels. Amongst artisanal foods produced in Africa some were shown to be of great interest like “attieke” and Gari two fermented cassava based staple food from the Ivory Coast and Nigeria [3,4]. Most of these are already exploited at industrial levels and are exported out of Africa [5-7]. We can mention also African black olive (*Canarium schweinfurthii* Engl.) presented as a novel ingredient for small scale traditional oil production [8] and number of other fermented products like fufu, Bikalga, kuri, naakia, Dawadawa and “bil bil” wish were investigated for their nutritional, antimicrobial, and probiotic aspects [9-11] and it was found that they all have interesting properties. However despite their importance most of indigenous African fermented foods and beverages have not received the scientific attention they deserve in the last decades. This is the case of “Lakh vzla” a food flavour enhancer used for notabilities amongst Mafa people from northern Cameroon. This paper aimed at describing the process production of this food enhancer and study microbiological and biochemical phenomenon which occur during the processing.

2. MATERIALS AND METHODS

2.1 Accessing Processing

The process production of “Lakh vzla” was followed according to selected individual producers (mainly women) and recording their comment and explanation step by step. This was completed by a focus group discussion [12] organised within five well-structured woman organisations surrounding the production area.

2.2 Determination of the Physico-Chemical Profile of Lakh vzla

2.2.1 Determination of ash

Ten grams of the raw bones and the fermenting “Lakh vzla” were weighed and transferred into a crucible of known weight. This was then ash-burned in a muffle furnace at 500ºC for 1 hr. The difference in weight was calculated according to the methods of Udo & Ogunwale [13].

2.2.2 Determination of pH

The pH was determined using a digital pH meter (HI 8418; Hanna instruments, Limena, Italy) calibrated with buffers at pH 4.0 and 7.0 (WTW, Weilheim, Germany). Samples were brought to room temperature then diluted in distilled water. Then the probe of the calibrated pH meter were introduced in the mixture. The pH values were read directly in the digital screen.

2.2.3 Determination of nitrogen

Twenty milliliter of concentrated sulphuric acid was introduced into the micro-kjeldahl flask containing 2 g of ground sample. Two Kjeldahl catalyst tablets were added and digested for 4 hr, cool overnight in a fume cupboard and the contents diluted with water to 250 mL. A distillation unit was then used and the percentage nitrogen was determined according to the Kjeldahl techniques of the AOAC [14].

2.2.4 Determination of moisture content

Six grams of the ground sample was dried in the air oven at 105ºC for 24hr. The sample was then cooled in desiccator. Further drying was done until constant weight was obtained. The moisture content was calculated according to the methods of Owoso & Ogunmoyela [15].

2.2.5 Determination of main metabolites

Collected samples were diluted in twice distilled water at 50%. The mixtures were centrifuged (10 min at 10000 g and 4ºC), and the supernatants

were filtered through a 0.22 µm pore-size membrane filter (Millipore). The filtrates were analysed on an Elmer Perkin liquid Chromatograph equipped with a hydrogen loaded ion exchange column (Bio-Rad Aminex HPX-87H, 300 by 7.8 mm). The mobile phase was 6mM H2SO4 with a flow rate of 0.8mL min-1 at 65ºC. Following metabolites were analysed using a corresponding internal reference for each of following putrescine, cadaverine, propionic acid, hydrogen sulfide and ammonia. After each metabolite the column was removed and washed with distilled water and rinse with the next internal reference.

2.3 Determination of the Microbial Profile of Lakh vzla

Samples were collected at each step during artisanal processing from interviewed women (producers). Each sample was divided into 500 g portions, wrapped in plastics bags and kept in refrigerated cooler bag, and then transferred to the laboratory for biochemical and microbial analysis. For isolation, each sample was divided into 100 g parts, then mixed with about 900mL of sterile 0.1% peptone water to form a solution. Serial dilutions were then made from solution and plated in agar based corresponding media [16]. Lactic acid bacteria were isolated on de Man Rogosa Sharpe (MRS) agar as described by Kandler & Sharpe [17]. Yeast and mould were isolated in potatoes dextrose agar as described by Jarvis [18]. Bacillus and other spore forming bacteria were isolated and identified according to Blanc, et al., [19] and bifidobacteria according to Munoa, and Pares method [20].

2.4 PCR restriction Analysis of 16s rDNA Method

All isolates were kept refrigerated at 4ºC in corresponding broth media until PCR analysis and identification. The identifications were performed by determination of 16S rRNA gene sequences of each isolate. A fragment of the 16S rRNA gene was amplified by PCR with conserved primers close to the 3- and 5- ends of the gene. The PCR products were directly sequenced (a single strand) by using a Taq dye-deoxy terminator cycle sequencing kit (Applied Biosystems, Foster City, Calif.) and an automatic DNA sequencer (model 373; Applied Biosystems). Identifications were based on a partial sequence (corresponding to positions 30 to 338 of the *E. coli* 16S rRNA gene) or a nearly full sequence (corresponding to positions 30 to 1521 of the E. coli 16S rRNA gene) comparisons. The closest known relatives were determined by performing database searches at GenBank by using BLAST program.

3. RESULTS AND DISCUSSION

3.1 Processing of "Lakh vzla"

The process production (Fig. 1) was started with the selection of bones. The preferred bones parts were said to be cartilaginous areas around articulations according to interviewed women. These selected bones were chopped and crushed into small pieces using stones or iron ax. This was mainly done by men and specially a butcher. The chopped and crushed bones were mixed with an amount of strong acid slurry (pH 2 to 3) basically stomach contain of cow or pig, citron juice or old acid artisanal beer and vines. This was allowed to step for 12 to 20 hr until the bones become plastic like smooth structure. The latter was washed with clean water and again mashed until a paste. The obtained paste was then mixed with pork or mutton grease and a small amount of and old "Lakh vzla" was used as starter culture. The obtained mixture was then poured into plastic bags and stored in humid and dark environment. The latter mixture was allowed to ferment for five to seven days according to the outside temperature. After fermentation, the mixture was rolled into balls then returned to the fermenting tanks for a further one to two days of retting.

Bone used for "Lakh vzla" is made of composite tissue composed of a hard tissue protein that is more resistant to degradation than other tissue proteins (collagen). This late serves as support for a mineral fraction that consists of hydroxyapatite a ground substance made of other organic compounds [21]. Hydroxyapatite and collagen are held together by a strong protein-mineral bond. This bond provides bone with its strength and its ability to remain long after the soft tissue has been degraded [22].

The biochemical process involved in degradation process of bone during "Lakh vzla" production was similar to that of diagenesis [23]. The first step in the process consisted of elimination of organic collagen fraction by the action of collagenases from microorganisms [24]. This collagenase may mainly be produced by isolated *Bacillus* sp. strains (Table 1). The produce collagenases break down protein into peptides which are subsequently reduced to amino acids which are involved in flavouring and particular

odour of “Lakh vzla”. Once the collagen has been removed from bone, the hydroxyapatite content is degraded by inorganic mineral weathering [25], meaning that important ions, such as calcium, are released as noticed in Table 1. Some bones used for “Lakh vzla” are mechanically broken down by physical breaking, decalcification, and dissolution during the first steps of the process (Fig. 1). The use of water during the production accelerates the process by leaching essential organic minerals from bone.

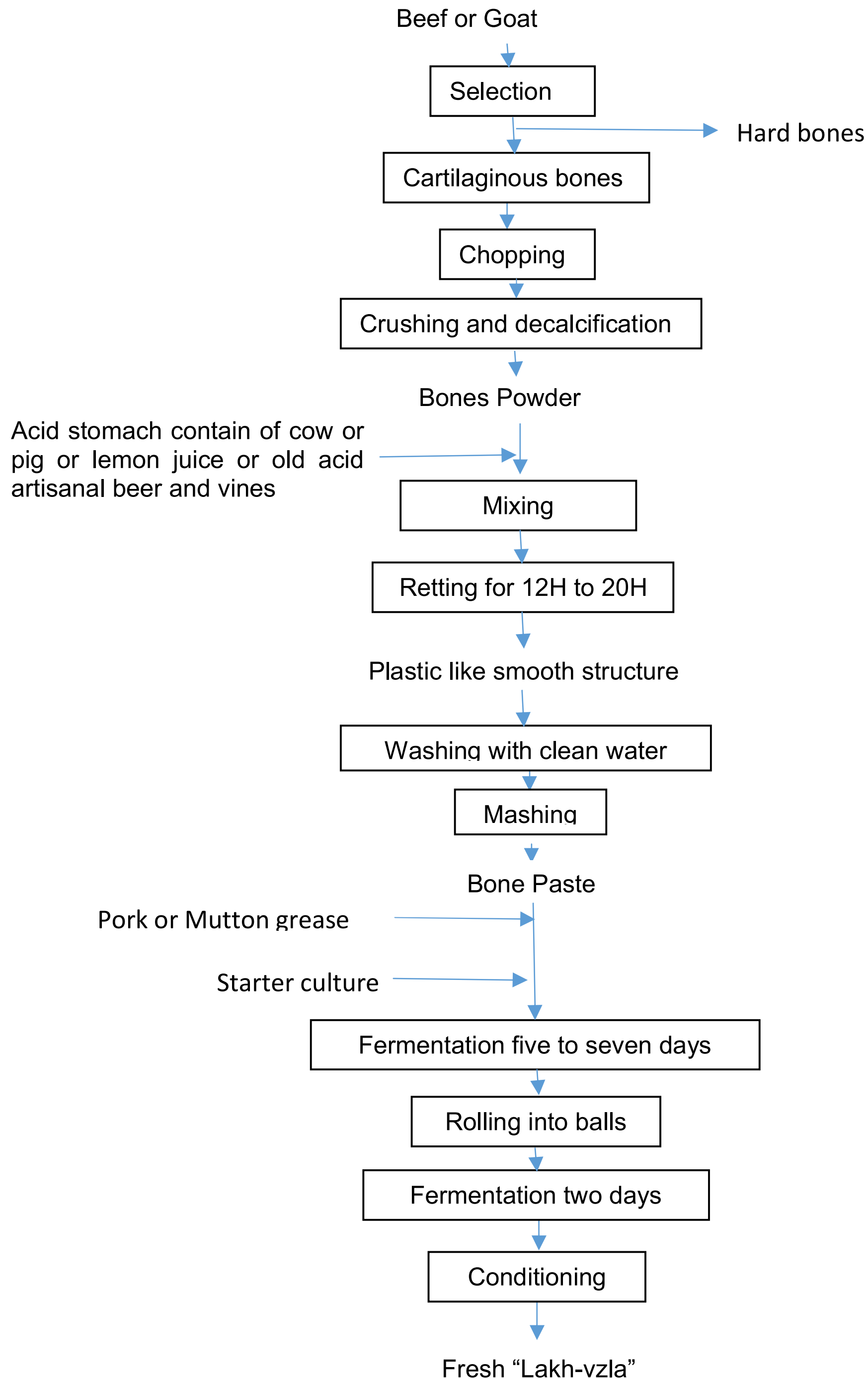

Fig. 1. Flow diagram showing process production of “Lakh vzla”

Table 1. Microbial profile and physicochemical parameters during

Step in the process/time	Microbial profile	pH	Ash g/100 g	Nitrogeng/100 g	Moisture (%DW)
Beef and goat bones	*Enterococcus faecalis*, *Escherichia coli*, *Staphylococcus* spp, *Streptococcus agalactiae Streptococcus sobrinus*, *B. stearorhermophilus*, *B. thermogfucosidasius*, *B. thermodenitrificans*, *B. pallidus*.	6.5±0.7[ac]	57±3[a]	8±2[a]	7.6±0.3[a]
Bone powder	*Actinobacillus actinomycetemcomitans Actinomadura madurae Bifidobacterium* sp, *Brucella melitensis*	5.0±1.0[ba]	89±8 [b]	7.8±0.7[b]	3.2±0.4[b]
After retting for 12 to 20 hr	*Clostridium histolyticum*, *Vibrio alginolyticus Capnocytophaga ochracea*, *Peptostreptococcus* spp	4.0±0.2[d]	82±15[c]	7.6±0.4[bc]	10±0.8[c]
Bone paste	*Clostridium histolyticum*, *Vibrio alginolyticus Proteus mirabilis*, Streptococcus mutans	4.1±0.5[d]	71±13[c]	7.5±0.5[c]	20±11[d]
Fermentation five to seven days (Balls)	*Clostridium histolyticum, Vibrio alginolyticus Proteus mirabilis Streptococcus mutans Pseudomonas aeruginosa, Serratia marcescens B. stearorhermophilus*, *B. thermogfucosidasius B. thermodenitrificans*	3.3±0.3[e]	80±11[dc]	4±1[de]	18±8[e]
Fresh “Lakh vzla”	*Clostridium histolyticum*, *Streptococcus mutans Bifidobacterium* sp., *B. thermodenitrificans Lactobacillus* sp., *Serratia marcescens*	3.2±0.1[e]	81±9[adc]	4.2±0.3[ed]	19±4[fed]

**Same case letters indicate no statistical differences between the analyzed parameters*

3.2 Amelioration of the "Lakh vzla": Semi Industrial Process

To make good "Lakh vzla", we advise to use for the first step (broth preparation): bones, cold water, and vinegar. These ingredients may be poured in a pot and the late mixture is allowed to sit at room temperature for about an hour to pull out calcium. The best bones to use for "Lakh vzla" processing is said to be knuckle and joint bones, probably because these parts contain the most connective tissues. Raw bones generally contain more gelatin than cooked bones, although this is not always the case. Optional ingredients used for "Lakh vzla" include animal heads and feet (these parts of the animal contain the most gelatin), vegetable scraps, and herbs. Next operation is to heat to a very slow simmer, and skim off any scum that should rise to the surface. The foam contains impurities that can give the broth aftertaste. It is then necessary to cover the broth and cook it and insuring that it doesn't boil. In case only fish bones are available, bake the broth for approximately 2 hours of slow simmering. In case one uses chicken bone, the baking time may increase from six to twenty hours. The beef bone should be cooked for 24 hours to 48 hours at light heat. When the final broth is obtained, allowed it to cool slightly, the stretched it and filled it into containers. Then starter culture made of old "Lakh vzla" or a two to three day fermented broth is added. Allowed fermentation for at least six to seven days but no more than ten days is applied. Then fermentation is stopped by refrigeration for about 3 to 4 days at 4ºC. The last day filter through a solid and clean nylon tissue. The solid part can be dried or roasted to give after crushin "Lakh vzla" powder. The extract liquid is also kept as liquid seasoning "Lakh vzla".

3.3 Microbial Profile

As seen in Table 1, there is a change of microbial profile according to the step of the process. In raw material, the isolated strains were *Enterococcus faecalis*, *Escherichia coli*, *Staphylococcus* spp, *Streptococcus agalactiae*, *Streptococcus sobrinus*, *Baccillus stearothermophilus*, *Baccillus thermogfucosidasius,* Baccillus *thermodenitrificans* and *B. pallidus*. The predominant strain at this stage were found to be *Enterococcus faecalis* (2.45 10^7 cfu/g). This strain formerly classified as a part of the group D *Streptococcus* system – is a Gram-positive, commensal bacterium inhabiting the gastrointestinal tracts of humans meaning that it can originate from human faeces thus hygiene indicator strain [26]. After powdering bone the microbial profile seems completely different with strains of microorganisms such as *Actinobacillus actinomycetemcomitans,* *Actinomadura* (*Streptomyces*) *madurae, Bifidobacterium sp, Brucella melitensis*. The predominant strain at this stage was found to be that of *Bifidobacterium sp*. (3.7 10^5 cfu/g) which was noticed earlier and this strain may produce collagenase an important enzyme during the process. The apparent disappearance of previously isolated hygiene indicator strains may eventually be linked to the drop of pH. During fermentation, we isolated and identified the following microorganisms: *Clostridium histolyticum*, *Vibrio alginolyticus*, *Proteus mirabilis*, *Streptococcus mutans*, *Pseudomonas aeruginosa*, *Serratia marcescens*. With Proteus mirabilis strains predominating in the first days of fermentation (2.7 10^6 cfu/g) followed by *Pseudomonas aeruginosa* (6.4 10^4 cfu/g) and *Serratia marcescens* (4.9 10^5 cfu/g). The microbial profile of the end product, "Lakh vzla" indicated *Clostridium histolyticum, Streptococcus mutans, Bifidobacterium* sp*., B. thermodenitrificans, Lactobacillus* sp*., Serratia marcescens*. The most predominant strain at this stage is *Bifidobacterium* sp. strain (2.4 10^4 cfu/g). Bacteria and fungi, play a role in bone degradation during "Lakh vzla" fermentation. They invade bone tissue and cause minerals to leach into the final product and thereby being more available as human nutrient.

3.4 Some Physico-chemical Parameters of "Lakh vzla"

Dry, fresh bone generally contains about 8% water that is loosely bound [27]. Turner-Walker, [28] It is found that that organic material in bone is approximately 22–23% by weight and 40% by volume [28,29]. Recorded value of around 35% seems a bit higher than that suggested by literature. This may be due to the fact that some sampled bone were not completely clean and flesh remain stuck in some bone area. About 90% of this organic material is made up of long fibrils of collagen which are highly organized, comprising three stretched helical amino acid chains which are themselves twisted into a triple helix [30]. As Collagen is characterized by a high glycine content, which makes up every third amino acid (33%), with high levels of proline and hydroxyproline, which together account for a further 20%. Each triplet is approximately 300 nm in length and 1.5 nm in diameter [31,32]. The

analysed sample of “Lakh vzla” showed that this food flavour enhancer is rich in all of the minerals in bone, especially calcium and phosphorus, and also magnesium, sodium, potassium, and sulphur. For this reason, “Lakh vzla” may also be an excellent supplement to the diet to support bone health. These minerals are present in a highly absorbable form. Anytime one or more of these minerals is deficient in the body, as is incredibly common nowadays, broth should be made a staple of the diet.

Putrescine and cadaverine may be produced by the degradation of amino acids and seems largely responsible for the foul odour of putrefying “Lakh vzla”. The acute and subacute toxicity of putrescine and cadaverine-were examined in wistar rats. Cadaverine were shown to have a low acute oral toxicity of more than 2000 mg/kg body weight. Putrescine had an acute oral toxicity of 2000 mg/kg body weight. The no-observed-adverse-effect level was 2000 ppm (180 mg/kg body weight/day) for cadaverine and putrescine [33] which is ten time the levels found in «Lakh vzla ». This may ensure potential consumers for eventual adverse effect.

As seen in Table 2. the level of propionic acid increased from around 100±12 ppm after retting to around 1500±87 ppm after fermentation. This clearly showed that fermentation step is responsible for propionic acid appearance in “Lakh vzla”. Propionates are naturally occurring substances in the normal diet and are produced by bacteria and occurs in various food and feed stuffs as a result of microbial production. Currently, propionic acid - propionates (E 280-283) are authorised food additives in the EU with maximal permitted levels (MPLs) ranging from 1000 to 3000 mg/kg in foods. Propionic acid inhibits the growth of mold and some bacteria at the levels between 0.1 and 1% by weight. Propionic acid produced is used as a preservative for both animal feed and food for human consumption. Another major application is as a preservative in baked goods, which use the sodium and calcium salts. The esters of propionic acid have fruit-like odors and are sometimes used as solvents or artificial flavorings [34].

Analysed “Lakh vzla” showed that it contained gelatin, which has many unique properties as increasing the digestibility of beans and meat [35]. Gelatin is also very soothing to the intestinal lining, and is good for anyone with digestive problems. Some of the many therapeutic uses of gelatin include support for peptic ulcers, diabetes, muscle disease, bone problems, infectious disease, and even cancer. It is nourishing and soothing for people who are sick. Some local consumers said “Lakh vzla” has a reputation for being the "artisanal penicillin". The gelatinous texture of “Lakh vzla” is considered locally as a "protein-sparer", meaning that one can get by with lesser protein when broth is used, because it enhances the body's utilization of protein [36]. For this reason, one can make an economical and nutritious soup with “Lakh vzla”, a small amount of meat or fish, and lots of vegetables.

We also isolated a substance called hyaluronic acid from “Lakh vzla”, which is a hydrophilic colloid. This means that it attracts gastric juices to the surface of food it is eaten or prepared with. All other cooked foods are hydrophobic, so they repel the gastric juices, impairing digestion. This problem can be eliminated with the addition of fresh “Lakh vzla” to cooked meals, or with the addition of homemade bone broth in soups and sauces. The wisdom of the ages is seen in the traditional practices of serving soup or salad as an appetizer, and the use of lacto-fermented salsas, chutneys, and fermented vegetables such as sauerkraut and kim-chee with meals consisting entirely of cooked food. In the preparation process of “Lakh vzla”, the important step of soaking the bones in water with a splash of vinegar before cooking (to pull the calcium from the bones) is skipped, so the mineral content is lessened.

Table 2. Some “Lakh vzla” metabolites

Step in the process/time	Principal metabolite (ppm)				
	Putrescine	**Cadaverin**	**Propionic acid**	**Hydrogen sulfide**	**ammonia**
Beef of goat bones	Nd	nd	nd	12±1	76±0,5
Bone powder	Nd	nd	nd	15±3	74±12
After retting for 20 h	Nd	nd	100±12	7±1	43±9
Bone paste	Nd	nd	126±22	6±0	40±8
Fermentation (Balls)	100±20	280±42	1000±43	17±4	106±13
Fresh “Lakh vzla”	150± 12	360± 33	1500±87	22±3	123±11

4. CONCLUSION

Fermented food condiments play an important role in the diets of many people in developing and a few developed countries. The rise in pH during production of these foods is due to the ability of the dominant microorganisms, Bacillus spp., to hydrolyze proteins into amino acids and ammonia. This study investigated “Lakh vzla” a popular food flavour enhancer amongst mafa tribe from farth North of Cameroon (central Africa). In this study, current knowledge about the principal microbiological activities and biochemical modifications which occur during the processing including nutritional, and antimicrobial aspects were presented.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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