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Kinetic study of some flavor and bioactive compounds during fermentation of *Parkia biglobosa*

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Abstract

Seeds of *Parkia biglobosa* also called African locust bean (ALBS) are used to produce a condiment by subjecting them to cooking, alkaline fermentation and drying. The objective of this work was to monitor the evolution of the composition of ALBS during spontaneous fermentation. Results showed that the seeds at initial time contained mainly proteins (45%) and lipids (36%). During fermentation, conversion of this initial composition took place to produce ammonium, free amino acids and short chain fatty acids (SCFA) that accounted for 2, 10 and 1.5% db respectively which explains the typical flavor of fermented ALBS. This release was also concomitant with biogenic amine production. Along with ammoniac and SCFA release, pyrazines were the major volatile organic compounds. Composition modulation was well represented by a Verhulst equation of which the dynamic could be modified by the initial composition of the seed, the pre-cooking step, and the fermentation conditions (temperature, aeration).

Key words: soumbala, aroma compounds, short chain fatty acids, biogenic amines, kinetics, Verhulst equation

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Practical applications

For nutritional and sustainability reasons, re-integration of vegetable in general and legumes in particular in our diet is a current trend. However, only dish that are appealing to consumers are concretely adopted. Fermentation of legumes is interesting in more than one way. It does not involve heat, so thermo-labile molecules can be preserved and the financial and energetic costs are low. Biological interesting molecules are produced from the metabolism of microorganisms and among them, volatile organic compounds that contribute to enhance the flavour of legume dishes. Soumbala is a meal and/or condiment obtained from the fermentation of African locust bean (*Parkia biglobosa*) seeds (ALB), a legume widely spread in west Africa. The final typical sensory properties are known to be due to the alkaline fermentation step by *Bacillus* species. This paper presents the kinetics of release of various compounds during spontaneous fermentation that may impact the sensory and nutritional properties of the final product. More precisely, the dynamic of the production of free amino acids, free fatty acids, volatile compounds and biogenic amines are presented and discussed to give insights into the succession of important reactions to control the sensory and nutritional quality of fermented ALBS. This paper shows that a lot can be done to better understand and control the quality of FALBS but also similar protein-rich legumes to modulate the quality of the final products.

Introduction

Legumes are a sustainable source of protein and other bio-active compounds that are sensitive to temperature. Fermentation is a non-thermal preservation process that can be applied to legumes. In addition, previous studies showed that due to microbiological activity, the fermentation step increased certain B-group vitamins and decreased anti-nutritional factors widely found in legumes such as alpha-galactosides or phytates (Adeyemo & Onilude, 2013; Granito et al., 2002). Some fermented products are traditionally made from legumes such as soybean, locust bean seed, chickpea, black gram or peanut (Reddy, 2018). A variety of microorganisms are involved: lactic acid bacteria, yeasts, fungus or *Bacillus* producing a variety of tastes, aromas, textures and flavors. The fermented legumes are widely used for flavoring in Asia or West Africa (Odunfa, 1988).

Among them, a typical and popular West African condiment, called *soumbala*, *netetu*, *afitin*, *iru* or *dawadawa* depending on the country, is made by *Bacillus* fermentation of a protein-rich legume, the African locust bean seed (*Parkia biglobosa*). The traditional process of soumbala production consists of three main steps: cooking the seeds in boiling water for 12 to 24h to soften the hard outer shell and facilitate dehulling, cooking the cotyledons, spontaneous fermentation, and final stabilization by drying (Dakwa et al., 2005; Odunfa, 1985; Yusuf & Rahji, 2012). This production is still done on a small-scale and therefore hardly meets consumer demand in terms of quantity as well as sanitary and organoleptic quality (Odunfa, 1985; Omafuvbe et al., 2004). Organoleptic attributes of fermented African locust bean seed (FALBS) are the result of the production of hundreds of chemical compounds during fermentation (Akanni et al., 2018; Ouoba et al., 2005). These numerous reactions are due to the metabolism of *Bacillus* species that hydrolyzes proteins to free amino acids, ammonia and other basic nitrogenous compounds. This results in an increase of pH and a so-called "alkaline fermentation", which contributes to the stability of the final product and also to its typical ammoniacal flavor (Akanni et al., 2018; Amoa-Awua et al., 2014; Ouoba et al., 2003). *Bacillus* not only produce proteases but a diversity of other extracellular hydrolases that also react with the seed carbohydrates and lipids which may contribute to the flavors and aromas development (Odunfa, 1985).

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3 87 Volatile organic compounds (VOCs) have already been studied in FALBS (Akanni et al., 2018;
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5 88 Ouoba et al., 2005). A total of 116 to 125 VOCs were identified, belonging, by order of
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7 89 quantities, to acids, pyrazines, nitrogen-containing compounds, aldehydes, alcohols, ketones,
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9 90 esters, alkanes, alkenes, benzenes, phenols, and sulfur-containing compounds. Depending on
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11 91 the relative concentration of the different VOCs, some consumers may or may not appreciate
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13 92 the product (Amoa-Awua et al., 2014; Dakwa et al., 2005). Indeed, as for strong aromatic
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15 93 products such as cheese or wine, the balance of the different molecules released and their
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17 94 control is of major importance on the sensory quality and acceptance of the end-product. To
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19 95 achieve this objective, knowledge of the impact of fermentation parameters on the
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21 96 production dynamic of the different volatiles is a first step. Akanni *et al.* (2018) and Ouoba *et*
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23 97 *al.* (2005) worked on the influence of *Bacillus* strains on the typology of final aroma
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25 98 compounds. Akanni *et al.* (2018) found that *B. subtilis* and *B. licheniformis* contributed to
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27 99 volatile acids, *B. cereus* to aldehydes and alcohols, and *B. amyloliquafaciens* produced sulfur-
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29 100 containing compounds. Another way to control VOCs release is to modify the processing
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31 101 conditions. Amoa-Awua *et al.* (2014) added humectant molecules like NaCl or glycerol during
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33 102 fermentation to limit *Bacillus* populations. They also tested the effect of steaming after
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35 103 fermentation. Both treatments applied significantly reduced the pungent odor because of a
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37 104 decrease in ammonia content. They found that the VOCs associated with the most undesirable
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39 105 sensory descriptors were ammonia, phenols, indoles, and volatile SCFA (Amoa-Awua et al.,
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41 106 2014; Delgado et al., 2011). On other fermented or ripened products such as cheese, authors
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43 107 found that SCFA had a major sensory impact because of their low odor threshold (Delgado et
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45 108 al., 2011; Faccia et al., 2018; Noronha et al., 2008).
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47 109 In addition to VOCs some non-volatile compounds may significantly contribute to the
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49 110 organoleptic properties of FALBS. Indeed, previous studies showed that FALBS contained
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51 111 significant amounts of free amino acids (AA) and FFA that can impact its sensory properties
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53 112 (Esse et al., 2021; Odunfa, 1985; Omafuvbe et al., 2004). Indeed, Zhang et al. (2020) showed
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55 113 that AA such as serine, aspartic and glutamic acids, glycine, alanine, proline, cysteine,
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57 114 methionine, and lysine, significantly and positively influenced the sensory descriptors of
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59 115 chicken broth and muscle (Zhan et al., 2020). Other bioactive molecules that may come from
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116 AA are biogenic amines (BA). Although BA are present in living cells and contribute to different
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118 physiological functions, high consumption via food can cause intoxication. Secondly,
elevated levels of biogenic amines can contribute to the pungent or objectionable odor of

fermented products (Cohen et al., 2014; Dekeirsschieter et al., 2009). The link between biogenic amines and the sensory profile of fermented products was studied in wine. It was proved that agmatine and β -phenylethylamine were correlated to a positive sensory profile, while putrescine, tyramine, histamine, cadaverine and spermidine were linked to negative descriptors (Ordóñez et al., 2017). Finally, FFA like myristic, palmitic, stearic, and eicosanoic acids can also affect the flavor of lipid-rich foods (Zhan et al., 2020). Alkaline fermentation of legumes in general, and that of African legumes in particular, is poorly studied. However, the use of fermented legumes contributes to food sustainability and flavor diversity. Therefore, it is important to gain insight into their production and into the reactions that takes place during fermentation that impact their sensory and nutritional quality. Because fermented African locust bean seeds is a condiment, the previous studies mainly focused on VOCs profile at the end of processing. However, a better understanding of volatile and non-volatile compounds release throughout the fermentation process would be very helpful for producers to better control the quality of fermented legumes. Therefore, the objective of this paper is to describe the kinetics of bioactive compound production during the spontaneous fermentation of ALBS.

Material and methods

Plant material

The harvest of African locus bean seeds takes place from March to May. Seeds are dried and are available on local markets till the next harvest season. The seeds used for this study were bought in the region of Khorogo, where most Soumbala production is located, in the north of Côte d'Ivoire.

Fermentation of African locust bean seeds

Before fermentation, ALBS were first put into boiling water at a ratio seed/water of 1/3 for 24h over a traditional wood fire to prepare dehulling. The seeds were dehulled in a laboratory mortar. Dehulled raw seeds were cooked in boiling water at a ratio seed/water of 1/1 for 1 hour. Spontaneous fermentation of 500 g of cooked cotyledons was done in jute bags placed in small baskets in a chamber regulated at 40 °C for 48 h. Samples were collected at 0, 12, 24,

36 and 48 h of fermentation. To stabilize them, fermented seeds were dried in an oven at 50 °C for 12 h. The whole process was repeated. A total of 17 samples were collected throughout the production process and more precisely: 3 samples of raw seeds, 4 samples of cooked seeds at time 0 of fermentation, 2 samples after 12, 24 and 36 h of fermentation and finally 4 at the end of 48 h of fermentation. All samples were dried and stored at -18 ° C.

Proximate composition

The dry matter content was determined by oven drying at 105°C to a constant weight. The nitrogen content of all samples was determined using the Kjeldhal method and a conversion factor of 5.85 was used according to AOAC 979.09. Lipid content was done by Soxhlet extraction according to AOAC 963.15. Total free fatty acids of the lipids obtained by Soxhlet extraction was determined by titrimetry according AOAC Cd 3d-63. Ashes were obtained gravimetrically after incineration of the sample at 550°C for approximately 7h according AOAC 923.03. Total carbohydrates were estimated by subtracting the measured protein, fat, ash, and water from the total weight.

Free and Total Amino Acid

Free amino acids were analyzed following the method used by (Moore et al., 1958), with modifications. Briefly, 15 mg of samples were weighed and placed in a sealable test tube. To this, 50 µl of internal standard Norleucine (25 µM) and 4.95 mL of citrate buffer (pH 2.2) were added. The solution was mixed for 1 h on a rotational shaker. For total amino acid determination, 10 to 20 mg of sample were weighed in a Schlenk tube and 50 µl of 25 µM norleucine and 450 µL of 4 M methanesulfonic acid were added. The tube was flushed with nitrogen, closed and heated at 150°C for 2 h. After cooling, 450 µL of 4 M NaOH was added to the hydrolysate, which was diluted to 5 mL with a loading buffer (citrate buffer at pH 2.2). Sample extracts for free and total amino acid analysis were filtered using a 0.45 µm membrane filter and injected into the amino acid analyzer (Biochrom 30+, Biochrom, France), using a lithium cation exchange resin column and ninhydrin as a detection compound. All total and free amino acid contents were determined except total tryptophan, which is the most fragile

amino acid and is destroyed by the extraction procedure. Amino acid standards were also run in a similar way as the samples.

Biogenic amines

Analysis of biogenic amines was carried out based on the procedure described in (Yoon et al., 2015). Briefly, 20 mL of 0.4 M perchloric acid were added to 5 g of the samples, and the mixture was homogenized using a vortex mixer (Vortex-Genie, USA), reacted in a cold chamber at 4 °C for 2 h, and centrifuged at 3000 ×g at 4 °C for 10 min. The supernatant was collected, and the residue was extracted again with an equal volume of 0.4 M perchloric acid. Both supernatants were combined, and the final volume was adjusted to 50 mL with 0.4 M perchloric acid. The extract was filtered then, derivatization of biogenic amines was carried out as follows: one mL of extract (or standard solution) was mixed with 200 µL of 2 M sodium hydroxide and 300 µL of saturated sodium bicarbonate solution. Two mL of dansyl chloride solution (10 mg/mL) prepared in acetone were added to the mixture, which was then incubated at 40 °C for 45 min. Residual dansyl chloride was removed by adding 100 µL of 25% ammonium hydroxide. After incubation for 30 min at 25 °C, the volume of mixture was adjusted with acetonitrile to 5 mL. Finally, the mixture was centrifuged at 3000 × g for 5 min, and the supernatant was filtered through 0.2 µm-pore-size filters (Millipore Co., USA). The filtered supernatant was kept at -25 °C before HPLC injection. An HPLC unit equipped with a UV-Vis detector (Dionex Ultimate 3000, France) was employed. An ACE C18 column (5µm particle size, 250 mm × 4.6 mm, ACE, France) was used for the separation of the biogenic amines, and the samples were eluted with a step gradient of ammonium acetate (0.1 M solvent A) and acetonitrile (solvent B) as the mobile phases at the flow rate of 1 mL/min. The program was set for a linear gradient starting from 50% of solvent B to reach 90% of the solvent at 19 min. The sample volume injected was 10 µL and monitored at 254 nm. Standard stock solutions of biogenic amines, putrescin, cadaverine, histamine and β-phenylethylamine were separately prepared at 10 mg/L concentration in distilled water.

Volatile compound

Firstly, 1.5 g of ground sample was placed and sealed in 10 mL headspace vials with 1.4 mg of the internal standard trans-2-octenal and extracted by headspace solid phase micro extraction (HS-SPME) (Corrales et al., 2017). The extraction was performed at 60°C with 15 min incubation and 30 min trapping and shaking using a polydimethylsiloxane/divinylbenzene fiber (PDMS/DVB 65 mm, SUPELCO, Bellefonte, PA, USA). The volatile compounds were identified using gas chromatography/mass spectrometry. A tandem gas chromatograph 6890/MSD 5973 N (Agilent Technologies, Palo Alto, USA) and a Gerstel autosampler MPS-2 were used. A non-polar capillary column DB-5MS (5% diphenyl, 95% dimethyl siloxane, 30 m × 0.250mm × 0.25 µm) and a polar capillary column DBWax UI (polyethylene glycol 30 m × 0.25mm × 0.25 µm), both from J&W Scientific (Folsom, CA, USA), were used with a carrier gas hydrogen flow rate of 1.2 mL/min. Then, fiber was desorbed at 250 °C in splitless mode and was eluted with the following temperature program: 3 °C per min from 40 °C to 170 °C, then 10 °C per min up to 240 °C and held for 10 min. Mass spectrum were recorded in EI+ mode at 70 eV within a range of 40 to 350 Da. Analyzer and source temperatures were 150°C and 250°C, respectively. Data were analyzed with Masshunter version B. 06.00 (Agilent Technologies, Palo Alto, USA). The identification of peaks was performed by comparing their mass spectra with those from the NIST 2011 (National Institute of Standard Technology) database. Co-injection on both columns of alkanes from C8 to C20 (Sigma-Aldrich, St. Louis, MO, USA) was used to calculate Kovats retention index (RI) for comparison with those found on the Flavornet, Pherobase and NIST websites.

Statistical analysis

One-way ANOVA was used to analyze the effect of fermentation time on the macronutrient composition in FALBS. If significantly different, means were further compared using Tukey’s test. One-way ANOVA was also done to select discriminant volatile compounds before principal component analysis (PCA). Statistical analyses were performed with Statistica® (StatSoft Inc., USA).

Kinetic modelling

Microbiological growth during fermentation is assumed to be logistic and that production of different bioactive compounds produced by microorganisms is often linearly correlated to population, therefore we chose the usual Verhulst logistic function to represent production (Peleg et al., 2007; Yang et al., 2011):

$$[X](t) = [X]_{\infty} \times \frac{1}{1 + L \times \exp^{(-k \times t)}} \quad \text{eq. (1)}$$

Where $[X]$ is the bioactive compound in g or mg.kg⁻¹ (DB) at the time t (h) of fermentation, $[X]_{\infty}$ is the asymptotic value of the curve that can be assimilated to the maximal concentration of X at the end of fermentation, k is the logistic growth rate or steepness of the curve while L refers to its sigmoid shape.

For each compound, the constants $[X]_{\infty}$, L and k were identified by non-linear regression thanks to the Levenberg-Marquardt minimization procedure using the Matlab® software (The Mathworks Inc., Natick, Mass, USA).

Parameter standard deviation

The standard deviations on identified parameters were determined via the method of bootstrapping simulations (Huet et al., 1992). The principle is the generation of a high number (500) of resamples of the observed dataset (and of equal size to the observed dataset) added with a Gaussian perturbation within the experimental standard deviations. The mean and standard deviations were estimated for each constant rate from the 500 values.

Results

Macro-composition evolution during fermentation

Raw ALBS were composed of 5% ashes, 25 % total carbohydrates, 25 % lipids and 45 % proteins, on a dry basis (DB). The cooking step resulted in significant leaking of carbohydrates that decreased from 25 to 15% DB while proportions of lipids increased to 36% and proteins remained stable. The cooking step also resulted in hydration of the dry seeds up to 60 to 70%, which was necessary for fermentation (Esse et al., 2021).

Fig. 1 represents the evolution of these macro-constituents on a DB during fermentation. In general, the composition of dry matter was quite stable. The major constituent being proteins,

then lipids and finally the rest of dry matter is composed mainly of carbohydrates and minerals. A slight change came from an increase of lipids from 35 to 42%. This increase was due to the preferential consumption of carbohydrates and proteins by microorganisms during fermentation and was already observed in other studies about ALBS fermentation (Azokpota et al., 2006; Esenwah & Ikenebomeh, 2008). However, within each nutrient category, especially proteins, transformations occurred because of microorganism actions.

Main changes of biological components during fermentation

Figure 2 a) b) and c) show the release of free amino acids (AA), ammonium and free fatty acids (FFA) respectively. For the three indicators, the logistic model of Verhulst fitted well the experimental data. Therefore, we can assume that their production was linked with the usual shape of microorganism growth of *Bacillus*. Production of AA, NH_4^+ and FFA started after about 10 h of fermentation. After an exponential increase, the maximal concentration was achieved after about 25 h for FFA, and 35 h for NH_4^+ and FFA. AA and NH_4^+ are released by protein degradation, while FFA comes from triglyceride hydrolysis. The maximal value of AA was almost 12 % which represented one third of the proteins. In their experiments, (Odunfa, 1985) found a level of 8% of AA at the end of fermentation. FFA reached 7% which represented 20% of the total lipids. The equation parameters of Verhulst are presented in Figure 3. The k values (equation 1) were higher for AA, then ammonium and lastly FFA which is consistent with our observations of proteins being consumed before lipids. The sigmoid curvature of free amino acids and ammonium release (L) was also higher than that of FFA. Therefore, we can say that during ALBS fermentation, the release of free amino acids occurred first followed by ammonium because of protein and amino acid catabolism and in a second time lipid catabolism occurred with the release of FFA. Odunfa (1985) showed that protease activity was higher than that of lipase. He also represented amino acid release of which the trend appeared logistic as well. Release of AA, NH_4^+ as well as FFA influence the flavor of fermented ALBS. Indeed, some AA can contribute to the umami taste (Lioe et al., 2010), while NH_4^+ induces the strong ammonia smelling characteristic of FALBS (Amoa-Awua et al., 2014). At last, FFA are major contributors to the flavor produced from lipolysis (Noronha et al., 2008). FFA and particularly palmitic, stearic and eicosanoic acids that are present in FALBS can produce a fatty flavor (Zhan et al., 2020). Previous studies showed that these three fatty acids were the most important after linoleic and oleic acids in ALBS (Esse et al., 2021). In addition to this direct

contribution to the sensory properties of FALBS, AA and FFA are precursors of other bioactive compounds shown in Figure 2 d) and e) which are biogenic amines (BA) and short chain fatty acids (SCFA). Those two compounds also behaved according to the Verhulst model and were produced after amino acid release. Indeed, BA and SCFA growth parameters were close with k equal to 0.16 and 0.15 respectively. Therefore, they could be considered as secondary products of amino acids. Mohamed et al. (2001) also showed that BA were produced after AA, which may be interesting as a tool to control fermentation or ripening time of Egyptian salted-fermented fish (Mohamed et al., 2001; Rabie et al., 2009). SCFA are fatty acids that have one to seven carbons. SCFA maximal content at the end of fermentation was 1.7%. SCFA could come from sugars, proteins and lipids (Akanni et al., 2018; Dursun et al., 2017). These molecules confirm the proteolysis and lipolysis that occur during ALBS fermentation and are known to contribute significantly to the acidic, sour, and pungent flavor of FALBS (Odunfa, 1985; Omafuvbe et al., 2004). In cheese, a rich protein and lipid product, SCFA contribute greatly to the flavor and its typicity and could be produced by anaerobic bacterial fermentation linked to the metabolism of *Bacillus* (Pasvolsky et al., 2014).

Release Kinetics of amino acids

The composition in amino acids of ALBS was presented in a previous article (Esse et al., 2021). The major amino acids were glutamic acid (Glu) at 20 %, followed by aspartic acid (Asp) at 10%, leucine (Leu) and lysine (Lys) at both 7%, arginine (Arg) and valine (Val) at 6%, alanine (Ala), proline (Pro), glycine (Gly), phenylalanine (Phe) at 5%, serine (Ser), isoleucine (Ile), tyrosine (Tyr), histidine (His), threonine (Thr) at 2 to 4%, and cysteine (Cys), methionine (Met) and ornithine (Orn) at concentrations <2%. Figure 4 shows the release of the different free amino acids. Among all amino acids of the legume, only 13 were significantly released and/or observable during fermentation. To be easily represented, the amino acids were presented on three different figures 4 a), b) and c) in g/100g DB. For all amino acids, the Verhulst function fitted the experimental data well which is consistent with previous observations (Odunfa, 1985). Among acid AA, Glu was the most produced reaching nearly 1.5 % DB which is consistent with the initial composition of the seed. This observation was already done and Odunfa (1985) stated that the release of Glu was particularly interesting in FALBS since Glu is a flavor enhancer. The author explained this high release by the ability of *Bacillus* species to accumulate Glu extracellularly. Cyst and Met were among the least produced which is

coherent with the low values of sulfur AA in legumes (Krishnan, 2005). However, Asp release was as low as the sulfur AA which was not consistent with the initial composition of the seed. Çalik et al. (2000) showed that *Bacillus licheniformis* was able to express the protease activity differently as a function of the oxygenation condition. In addition, the types of amino acids that were excreted to the fermentation medium also depended on the oxygenation conditions. The least release of amino acids of the aspartic acid-group could be explained by the need of this AA in *Bacillus* cells and a low asparagine synthetase expression (EC. 6.3.1.4) or high-aspartate kinase (EC. 2.7.2.4) activity (Çalik et al., 2000). Another non-observable AA was Arg, although it was the fifth in terms of initial quantity. However, Çalik et al. (2000) did not observe its non-excretion. With Cys (Fig. 4a), Phe and Lys (Fig 4c), Leu, Ile, Val, and Trp (fig 4 b) are essential amino acids. Leu, Val, Phe, and Lys releases were close to 1% at the end of fermentation. As their initial content in the seed was about 3% DB, nearly 30% of the amino acids were in their free form at the end of fermentation which may be beneficial for a good assimilation of these essential amino acids (Ito et al., 2018; LU et al., 2017). Odunfa (1985) also observed a high release of valine. Recommended daily amounts for these essential AA are 25 to 40 mg/kg/day as a function of AA (FAO/WHO/UNU, 2007). Therefore, the need for people weighing 60-80 kg is 1.5 to 3 g/day. Assuming one portion of this condiment weighs 20 g, a meal can provide 20 to 30% of recommended daily intake. The non-essential AA Tyr also reached 1% while His and Orn reached 0.5%. At the end of fermentation, these AA were at 80, 40 and 60 % in their free form respectively. Therefore, these compounds may be not crucial for *Bacillus* cell metabolism or were favorably excreted.

Release Kinetics of biogenic amines

The main BA found and presented in Fig. 4d were by order of importance spermidine (Sper), β -phenylethylamine (Phen), histamine (Hist), putrescine (Put), and cadaverine (Cad). Sper comes from from Arg, Phen from Phe, Hist from His, Put from Arg and Cad from Lys. The final levels were 200, 90, 65, 30, 40 ppm respectively leading to a total close to 400 ppm (Fig. 4). This total value reached the limit defined by the European regulation for products having undergone an enzymatic maturation treatment. Therefore, BA production must be monitored carefully during ALBS fermentation. The high content of Sper may be due to the high conversion from Arg, and may explain the absence of this AA in Fig 4. An intermediary compound of this conversion can be Put. In cured ham, Arg content was low due to its

hydrolysis to ammonia and Orn, followed by decarboxylation to Put that was most largely present in aged hams (Virgili et al., 2007). Also, it was suggested that the amounts of Put and other BA in hams could be inversely related to the levels of their amino acid precursors and that some BA generation may be regarded as the last step of the decarboxylation-deamination mechanism starting with Arg (Alfaia et al., 2004). For the kinetic aspect, the most rapidly produced free AA were Phe, His, Met, Glu, and Ile with $k > 0.5$ (Figure 3). Regarding BA, the most rapidly produced were Hist, Cad, and Put respectively and their maximal concentration was reached at the early stage of fermentation, that is to say after about 10 hours. Hist production was highly correlated to Hist release. Cad was produced more rapidly than its precursor but rapidly reached a maximum. Put was probably produced very rapidly from Arg, but the quantity of this BA was low, probably due to its rapid conversion to Sper. Phen and Sper were produced in the second part of fermentation and their maximal value was reached after 30 hours of fermentation. It is important to remark that Sper and Hist did not fit Verhulst model after reaching their maximal content. Indeed, their content decreased suggesting a potential degradation. This reaction may be conducted by microorganisms including certain strains of *Bacillus* (Alvarez & Moreno-Arribas, 2014; Lee et al., 2016).

Release Kinetics of Short chain fatty acids

The most odorant SCFA are present in Fig 5a. They were composed of formic (C1), acetic (C2), butyric (C4) and the branched 3-methylbutanoic acid also called isovaleric acid (C5). Their respective odor threshold in air in ppm is $8, 5 \cdot 10^{-3}, 3 \cdot 10^{-3}$ and $8 \cdot 10^{-5}$ respectively. Formic acid odor is described as sharp acetic and fruity fermented, that of acetic acid, sour vinegar, while butyric acid odor is cheesy, rancid and that of isovaleric acid, pungent. Less odorant SCFA are on Fig 5b with lactic, oxalic, malic, tartaric, and citric acids (C2, C3, C4 and C6 respectively). In addition to carboxylic function, lactic, malic, and tartaric acids have one or two alcohol functions and oxalic and citric acids are respectively di- and tri- acids which may contribute to decreasing their volatility and therefore increasing their odor threshold. An interesting point is that the odorant SCFA were produced during fermentation while the others decreased (tartrate and citrate being the most abundant in the raw seeds) or increased and then decreased (oxalate, malate, and lactate). SCFA may have different origins. Butyric acid may come from FFA like in cheese (Faccia et al., 2018; Poveda, Justa María et al., 2008). Acetic acid may come from the oxidation of ethanol during fermentation while Isovaleric acid (3-

methylbutanoic acid) from branched amino acids like valine, leucine, and isoleucine. Among volatile acids, 2-methylbutanoic and 3-methylbutanoic acids were found to contribute largely to the typical aroma of cheese or fermented ALBS (Akanni et al., 2018; Poveda, Justa María et al., 2008). Also, the Verhulst function described odorant SCFA release well. The first acids produced were acetic and formic acids at a slow rate followed by isovaleric and then butyric acids that exhibited a more logistic shape (higher L parameter) and slightly higher rates of formation (Figure 3). This order of release is concomitant with the successive consumption of carbohydrates, proteins and lipids as substrates for *Bacillus*. Therefore, SCFA are prevalent compounds released throughout ALBS fermentation but their individual prevalence could be modulated by the macro-composition of ALBS and by fermentation time. This modulation could markedly influence odor but also taste of FALBS.

Evolution of the different VOCs families

A total of 111 volatile organic compounds (VOCs) were collected from the different samples: raw seeds, and cotyledons during different times of fermentation which is consistent with the number of 116-125 found in previous studies on similar products (Akanni et al., 2018; Ouoba et al., 2005). A total of 13 pyrazines, 9 aldehydes, 11 ketones, 15 esters, 16 acids, 15 alcohols, 1 alkene, 1 amine, 2 pyridines, 8 benzenes, 2 phenols, 2 sulfur compounds, 6 amides, 1 furan and 9 others were found. 60 of the most frequent VOCs constituting the 'volatolom' of our samples of FALBS are presented in Table 1. These compounds were found in other protein-rich products like beans, cheese, milk, shrimp, or cured fish such as anchovies or fermented products such as coco beans, wine, or free amino acid-rich products such as mushrooms (Aisala et al., 2019; Akanni et al., 2018; Amoa-Awua et al., 2014; Dehaut et al., 2014; Dursun et al., 2017; Kouakou-Kouamé et al., 2020). During cocoa bean fermentation, there is a succession of VOCs families as a function of fermentation time due to microbial succession of a wide range of yeasts, lactic acid and acetic acid bacteria (Kuhnert et al., 2020; Schwan, 1998). In the case of ALBS fermentation, flora is supposed to be more uniform and mainly composed of *Bacillus* conducting an alkaline fermentation. Therefore, the study of the release kinetics of VOCs in FALBS may contribute to explaining the metabolism behavior of *Bacillus* and its relationship with the final aroma.

Figures 6 and 7 present the evolution of the different families of VOCs during production of fermented ALBS. The main constituents of raw seeds were alcohols (~30%), acids and esters.

After cooking, a marked change occurred with an increase of pyrazines from 10 to nearly 60 % of VOCs. This increase was certainly due to the Maillard reactions during cooking but also the leaking of some hydrophilic or thermosensitive compounds like alcohols and esters while the acids remained stable. After fermentation, a second marked change was observed with the prevalence of acids that account for more than 45% of the VOCs. Pyrazines still remained at a high concentration as can be observed in Figure 5a). Their production was not monotonous. Indeed, their content increased at the beginning of fermentation but decreased after 12 h so their final quantity was not significantly different from that at the beginning. The total of VOCs increased significantly during fermentation ($p < 0.05$) and were strongly positively correlated to the production of acids ($p < 0.05$). As can be seen in Fig 5b), another category of product that rose markedly during fermentation were amides ($p < 0.05$). The other compounds: alcohols, aldehydes, esters, ketones, and furans, decreased at the beginning of fermentation (specially for aldehydes, esters and ketones) but were produced after 24 h. However, their final content was still inferior or not significantly different to $t=0$ h. Sulfur compounds were in small quantity and did not evolve significantly.

Succession of VOCs during fermentation

To be more specific about the different molecules that evolved significantly during FALBS production, ANOVA analysis was carried out for all VOCs followed by PCA only on the molecules that discriminated the fermentation time. The results of PCA are presented in Fig. 8. The projection of different samples showed that PCA enabled the discrimination of raw seeds (RS) from the cooked cotyledons before fermentation (0 H). The typical VOCs that were positively correlated with the raw seeds were: ethanol, hexanol, carbon disulfide, butanal and 4,6,6-trimethylbicyclo[3.1.1]hept-3-en-2-one. This last molecule is also called verbenone and has a camphoreous odor (Table 1). Alcohols are typical of the raw seeds and among them 1-hexanol which is responsible for green notes than can be found in peas for instance (C. Zhang et al., 2020).

These compounds were lost significantly during cooking and may therefore contribute only slightly to the flavor of the final product. However, some VOCs were positively correlated with $t=0$ hours and therefore may have appeared during cooking. Among them, one pyrazine, 2,3-Dimethyl-5-ethylpyrazine that has a strong roasted aroma, could have come from the long cooking period (Qian & Reineccius, 2002). We also found xylene which is an aromatic

hydrocarbon and may be considered as contamination arising from wood-cooking (Ciecierska, 2020). Lastly, 4-methanol-himidazole is a late Maillard compound (Licht et al., 1992). These two last compounds are cyclic neo-formed compounds produced because of the very long cooking time that may have an adverse effect on health at high concentrations.

The compounds positively correlated with the first hours of fermentation were aldehyde (benzaldehyde and 2,5-dihydroxybenzaldehyde), esters (ethyl acetate), ketones (acetoin, 2,3 butadione, 2-decanone and 2-octanone), 2-pentyl-thiophene and 2-pentyl-Furan. 2-pentylfuran and 1-octen-3-ol compounds are known for contributing to the green and beany aroma of cooked beans (Owens et al., 1997). They were also found in sugar-rich fermented products such as grape wine, alcoholic beverages made from sorghum and particularly fruit wines involving yeasts (Feng et al., 2015; Song et al., 2019; H. Zhang et al., 2021). Therefore, these compounds may come from the cooking process and the early stage of fermentation when *Bacillus* consume sugars and provide fruity notes. Owens et al. (1997) reported that *Bacillus subtilis* when grown in sugars excrete alpha-acetolactate that is later decarboxylated to acetoin which is consumed when carbohydrate depletion occurs. He also noticed that the other ketones produced may be the result of lipid and/or amino acid degradation. However, these compounds have a relative high odor threshold so they may only contribute slightly to the aroma of FALBS.

The other group that appeared during fermentation were pyrazines. Their maximum content was achieved between 10 to 15 h of fermentation. The main pyrazines by order of quantity were 2,5-dimethyl-pyrazine, methyl-pyrazines, ethyl-pyrazine, 2-ethyl-6-methyl-pyrazine, and 2,6-dimethyl-pyrazine. Pyrazines in general and 2,5-dimethyl-pyrazine in particular were also found during *Bacillus* fermentations (Akanni et al., 2018; Besson et al., 1997; Ouoba et al., 2005; Owens et al., 1997). Owen et al. (1997) also noticed that the free amino acids, the alkaline pH and the incubation temperature (40 °C) favored the production of pyrazines. Ouoba et al. (2003), during fermentation in controlled conditions in the laboratory with different *Bacillus* starters, found that the profile of pyrazines, ketones, aldehydes and esters was dependent on the *Bacillus* species. In our case, we observed that the amount of pyrazine significantly decreased at the end of fermentation until it reached the initial level just after cooking. This decrease was also noticed but not explained by Owen et al. (1997). Therefore, the roasted notes of the final product due to pyrazines may depend on the fermentation time.

Contrary to pyrazines, acids were released constantly throughout fermentation. The main acid in quantity was 3-methyl-butanoic acid (isovalerate) as observed previously, followed by 2-methyl-butanoic acid. In addition, with these two volatile acids, we found 2-methyl-2-butenic acid also called tiglic acid. It is supposed to be formed from isoleucine and presents ripe fruit notes (Y. Zhang et al., 2018). Simultaneously with acids, esters were formed. Akanni et al. (2018) noticed that esters have been known to constitute a major volatile compound in African fermented condiments. The esters are presumably the consequence of chemical reactions between acids and alcohols and produce floral and fruity notes. Logically, the main esters found were that of the main acids, i.e., 3-methyl-butanoic ester, 2-methyl-butanoic ester, and acetic acid-methyl ester. The other secondary products released were amides that were correlated to the last hours of fermentation. The main amides were acetamide, propanamide, butanamide, 2-methyl-propanamide and 3-methyl-butanamide. These molecules may come from the reaction between free amino acids and acids and were observed in cocoa beans and wine (Kuhnert et al., 2020; Schueuermann et al., 2016). Therefore, acids are involved in different reactions with alcohols and free amino acids which may explain the slight decrease of certain acids like formate or malate at the end of fermentation as observed in Fig 5a and b. Lastly, two other VOCs were found at the very final stage, which were phenol, a degradation product of tyrosine, and 2-piperidinone found in fermented bean (Kim & Chung, 2008; Mathus et al., 1995). Phenol is an irritant and considered objectionable while 2-piperidinone, is ammoniacal and pepper-like (Maraval et al., 2008). Again, specific monitoring of the last hours of fermentation could be of interest to control the concentration of these two last compounds.

Conclusion

This paper showed the release of different bioactive compounds during spontaneous FALBS production that have sensory, nutritional and toxicity impacts. During fermentation, VOCs number was multiplied by a ten-fold which confirm the very important impact of this step on the final quality of FALBS. This is because ALBS is a legume rich in proteins and lipids and because *Bacillus* species involved in this fermentation are able to metabolize them with their proteases and lipases. The results also highlighted the important kinetic aspects of this fermentation. The main early products are a result of protein catabolism and are free amino acids with glutamic acid presenting the highest release and some biogenic amines like

cadaverine, putrescine and histamine. In a second phase, SCFA and FFA releases occurred. Particularly, the cleavage of branched-chain amino acids produced isovaleric acid which was the main acid produced. The odorant butyric acid was produced later and at a lower content certainly due to triglyceride hydrolysis. Therefore, we can conclude that the first 20 hours of fermentation of ALBS lead to maximal contents in acids contributing significantly to the “sour, pungent and cheesy” notes of FALBS. During this phase, pyrazines were also produced in high certainly giving roasted notes to FALBS. Lastly, esters, amides and phenol were produced from complex reactions between amino acids, alcohols and acids produced in the previous phases. Maximal ammonia content was achieved during this phase increasing the final pH of FALBS.

The perspectives of this research will be to work firstly on the raw material. Indeed, raw seed composition and their pre-treatment (cooking) are important to monitor the proportion of carbohydrates, proteins and lipids that are the substrates of fermentation. Secondly, it would be important to deepen the link between *Bacillus* species growth and the bioactive compounds release during fermentation. To do so, a controlled fermentation should be done and *Bacillus* population, enzyme activities, AA, BA, SCFA, and VOCs should be monitored as a function of fermentation time. Lastly, the link between fermentation conditions (temperature, aeration) and these dynamics would be very crucial to make in order to understand the possible levers of bioactive compounds modulation in the final product.

Conflicts of interest statement

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Figure and Table list

Figure 1. Evolution of macro-constituents during fermentation of ALBS

Figure 2. Release in g/100 g DB of a) free amino acids (AA), b) ammonium (NH_4^+), c) free fatty acids (FFA), d) biogenic amines (BA), e) short chain fatty acids (SCFA) during ALBS fermentation. Error bars represent the standard deviation ($n = 3$) and lines represent the modelled data.

Figure 3. Verhulst equation parameters for all compound releases during fermentation of ALBS

555 **Figure 4. Release of a), b), c) free amino acids and d) biogenic amines during ALBS**
556 **fermentation. Error bars represent the standard deviation (n = 3) and lines represent the**
557 **modelled data.**

558 **Figure 5. Release of a) odorant SCFA and d) less odorant SCFA during ALBS fermentation.**
559 **Error bars represent the standard deviation (n = 3) and plain lines represent the modelled**
560 **data.**

561 **Figure 6. Evolution of the different VOCs according to the chemical family during fermented**
562 **ALBS production**

563 **Figure 7. Evolution of a) total VOCs, acids and pyrazines and b) other VOCs families during**
564 **ALBS fermentation**

565 **Figure 8. PCA on molecules that evolve significantly during FALBS production (RS: raw seed)**

566 **Table 1. Most frequent VOCs produced during fermented ALB production**

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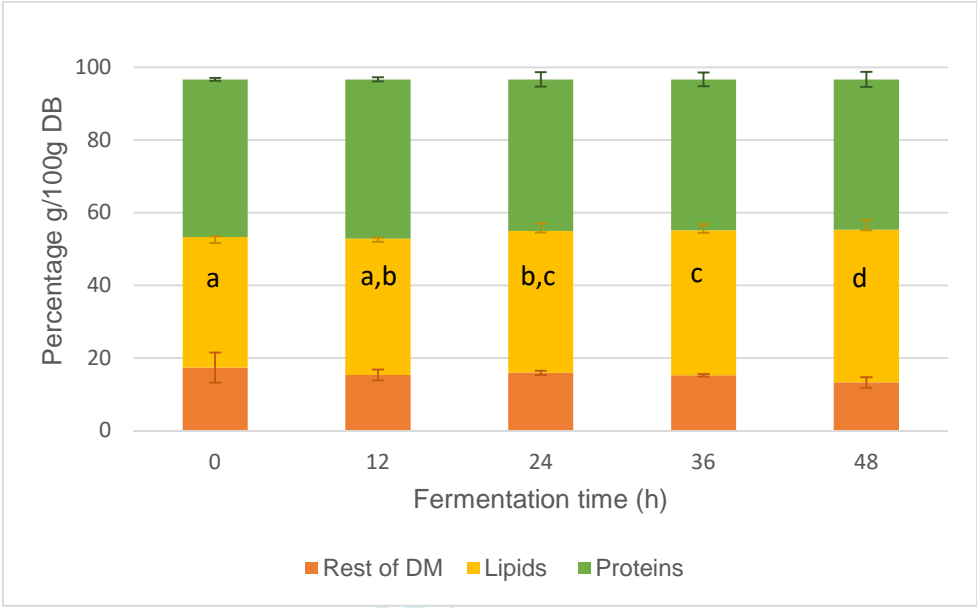
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Values bearing different letters for the same parameter are significantly different ($p<0.05$).

Figure 1. Evolution of macro-constituents during fermentation of ALBS

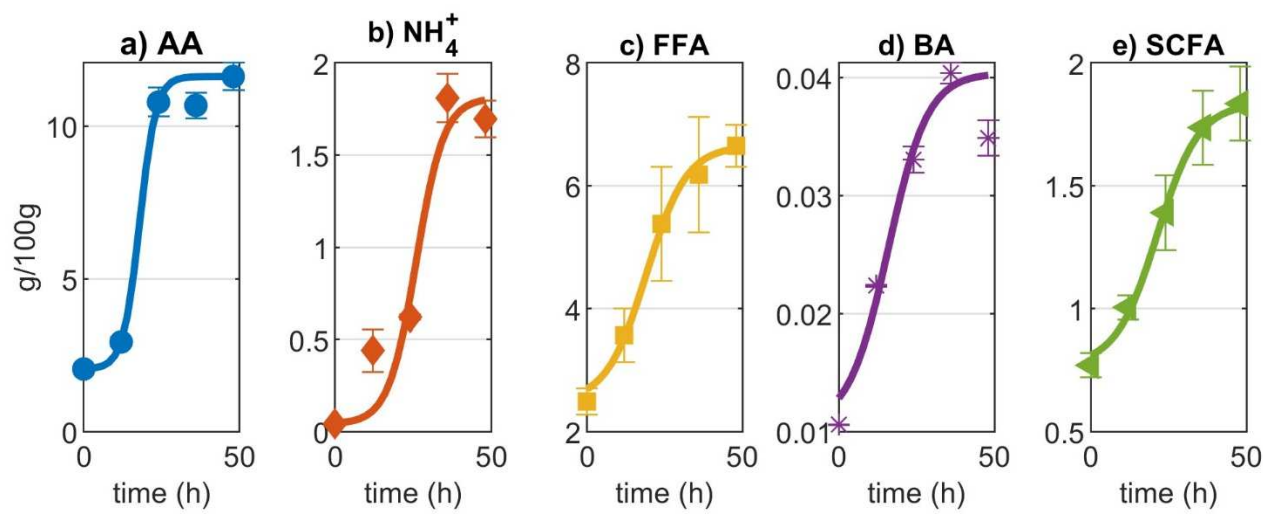


Figure 2. Release in g/100 g DB of a) free amino acids (AA), b) ammonium (NH_4^+), c) free fatty acids (FFA), d) biogenic amines (BA), e) short chain fatty acids (SCFA) during ALBS fermentation. Error bars represent the standard deviation (n = 3) and lines represent the modelled data.

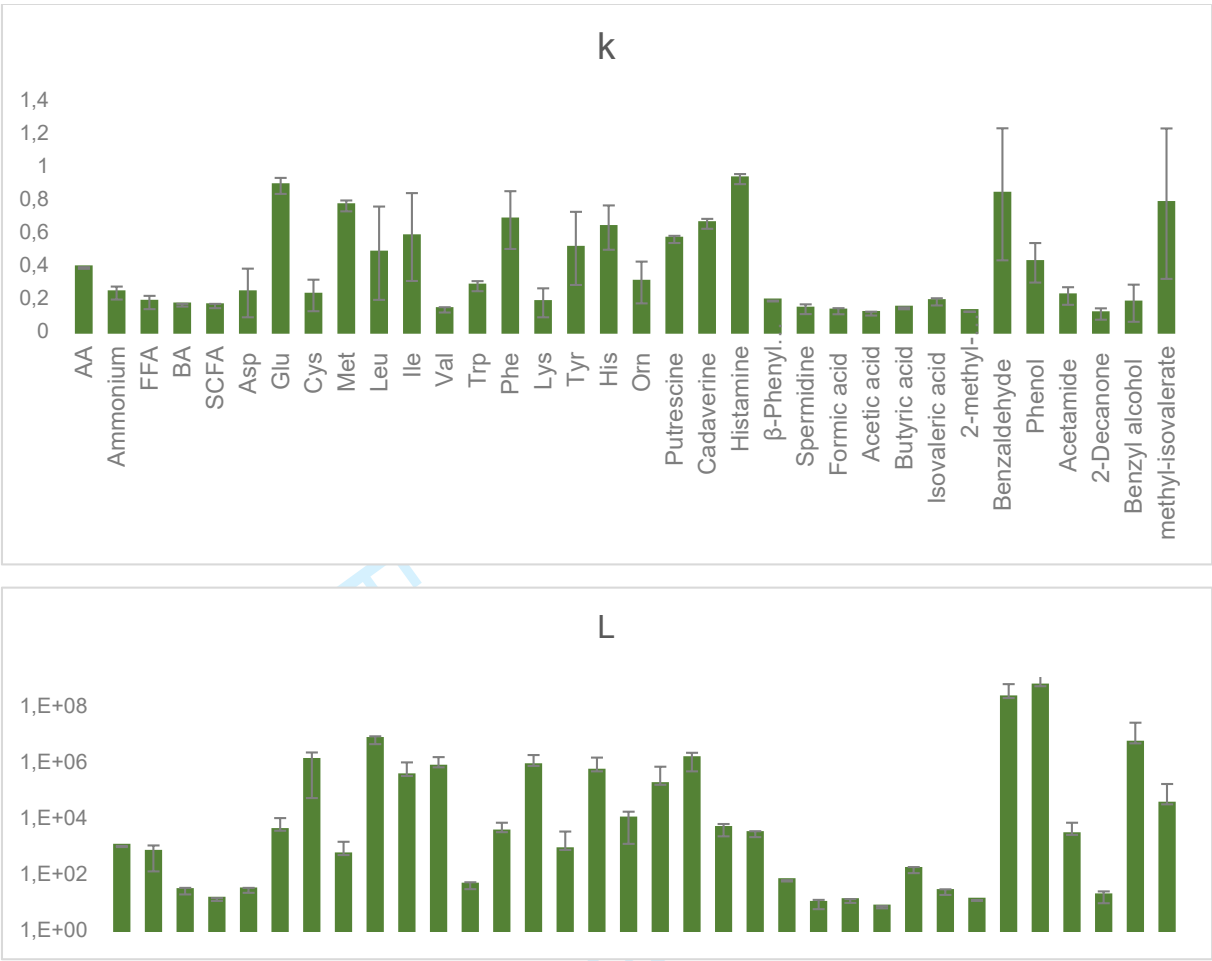


Figure 3. Verhulst equation parameters for all compound releases during fermentation of ALBS

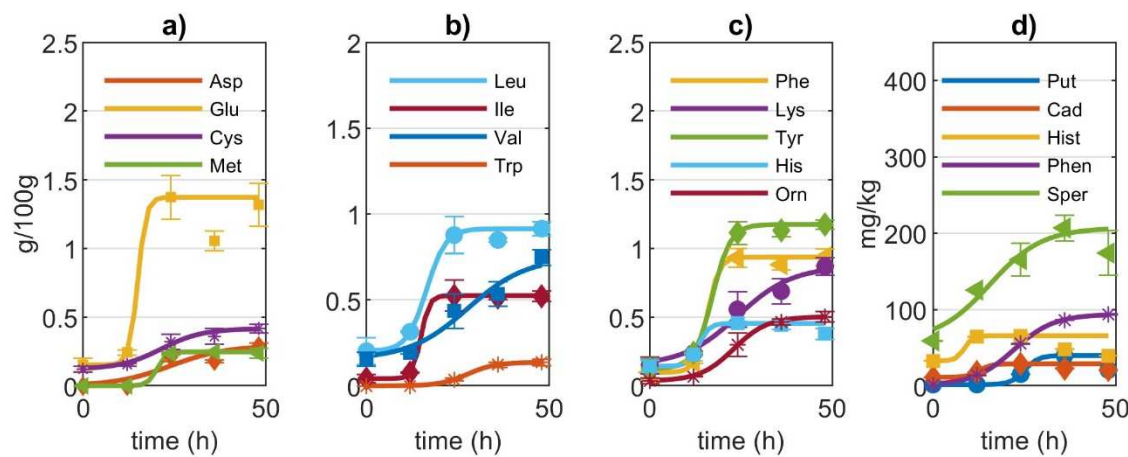


Figure 4. Release of a), b), c) free amino acids and d) biogenic amines during ALBS fermentation. Error bars represent the standard deviation (n = 3) and lines represent the modelled data.

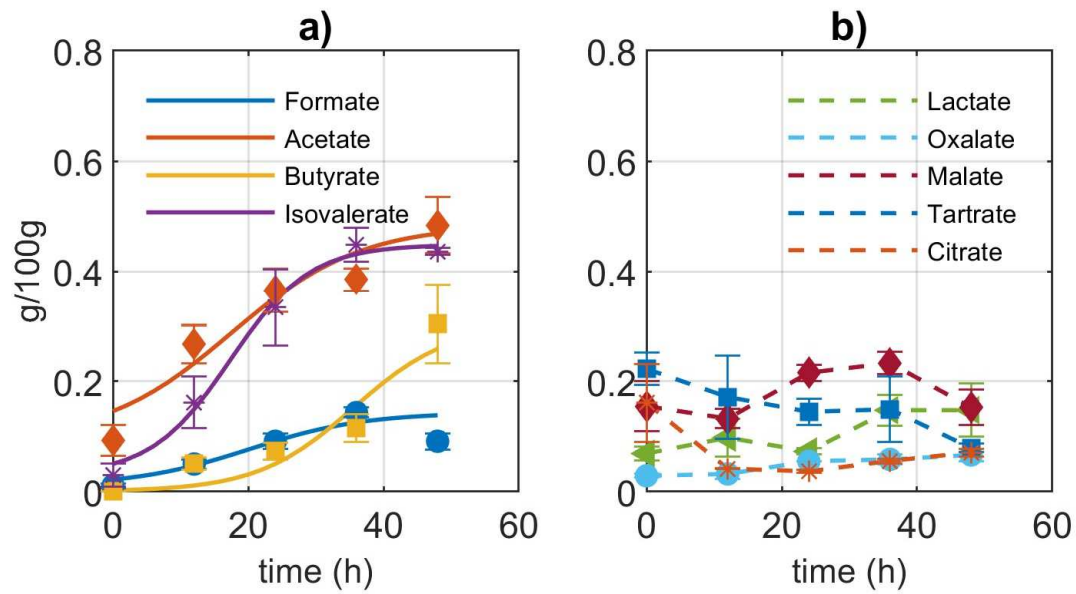


Figure 5. Release of a) odorant SCFA and d) less odorant SCFA during ALBS fermentation. Error bars represent the standard deviation (n = 3) and plain lines represent the modelled data.

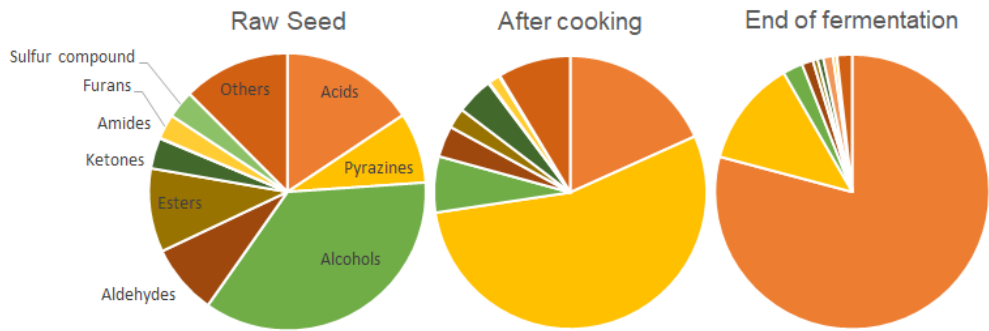


Figure 6. Evolution of the different VOCs according to the chemical family during fermented ALBS production

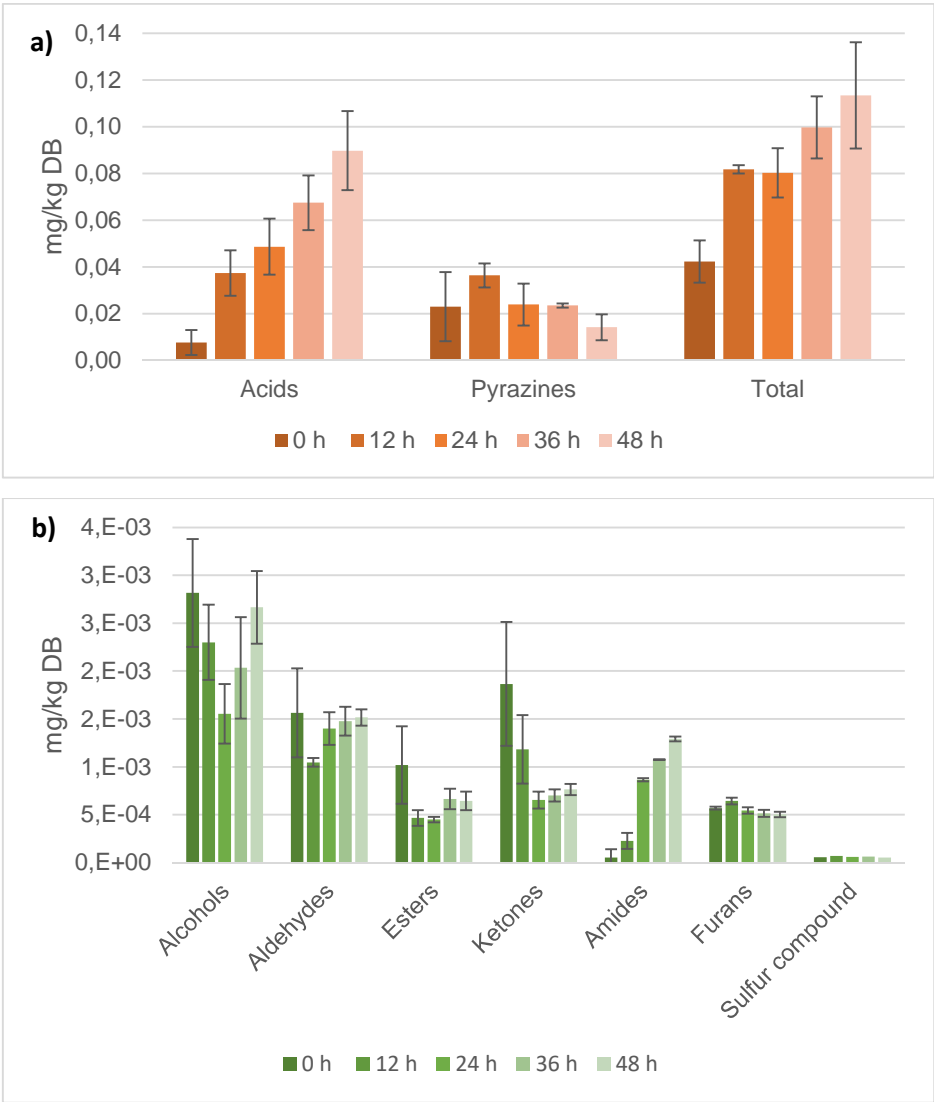


Figure 7. Evolution of a) total VOCs, acids and pyrazines and b) other VOCs families during ALBS fermentation



Figure 8. PCA on molecules that evolve significantly during FALBS production (RS: raw seed)

	<i>Odor</i>		<i>Assumed bioreaction origine</i>	<i>Odor threshold (µg/kg) in water</i>	<i>Product</i>	<i>References</i>	<i>Present in raw ALBS</i>
<i>Acids</i>	3-methyl-butanoic acid (isovaleric acid)	acidic, sour, pungent,	produced from branched-chained amino acids such as valine, leucine, and isoleucine during fermentation	120	Bambara bean	Akanni (2018)	
	2-methyl-butanoic acid			100	Bambara bean	Akanni (2018)	
	2-methyl butenoic (tiglic acid)	Ripe fruit			Beverage fermented with shiitake	Zhang (2018)	
	2-methyl-propanoic acid (isobutyric acid)	Meat, cheese	-		Adjuevan	Kouakou-Kouame (2020)	
	Ethanoic acid (Acetic acid)	sour, vinegar note	Oxidation of ethanol		Bambara bean	Akanni (2018)	
	Butanoic acid (butyric acid)	rancid, cheesy			Cheese	Delgado (2011)	
	Propanoic acid (Propionic acid)	pungent acidic cheesy vinegar	hydrolysis triglycerides by lipases		Milk	Dursun (2017)	
<i>Pyrazines</i>	2,5-dimethyl-pyrazine	chocolate, roasted nut	from free amino acid liberation and heating	800			
	trimethyl-pyrazine	roasted, burnt notes		400	Bambara bean	Akanni (2018)	
	2,6-dimethyl-pyrazine	chocolate, roasted nut, fried potatoes		200			
	tetramethyl-pyrazine	nutty, musty, chocolate		1000			
<i>Alcohols</i>	1-Hexanol	Herbal, fusel oil, alcoholic	-	20000	*		yes
	Phenylethyl Alcohol	Floral, rose, honey	-	20000	*		
	1-Octen-3-ol	mushroom and fermented-like odor	oxidation of linoleic acid		Bambara bean, Adjuevan	Akanni (2018), Kouakou-Kouame (2020)	
	1-Octanol	waxy, paint jar			mushrooms	Aisala (2019)	
	3-Octen-1-ol	fatty fruity herbal					
	Ethanol	Alcoholic	fermentation of sugars		bambara	Akanni (2018)	yes
	Benzyl alcohol	pleasant, fruity	-		bambara	Akanni (2018)	
	2-Octen-1-ol	Dust, cement, mushroom	reduction of aldehydes		Milk	Dursun (2017)	
	2,3-Butanediol	rubber, fruity, creamy, and buttery	produced from branched-chained amino acids		Bambara bean	Akanni (2018)	yes
<i>Aldehydes</i>	Benzaldehyde	pleasant, sweet, aromatic note	cysteine heating, heat generated compound, amino-acids catabolism	350	Bambara bean milk, anchovies	Akanni (2018), Dursun, Dehaut (2014)	
	Butanal (butyraldehyde)	Aldehydic, fruity	oxidative cleavage of lipids		African locust bean	Quoba (2015)	yes
	Hexanal	green odor, fat		4.5	bambara, African locust bean, anchovies, Adjuevan	Akanni (2018), Quoba (2015), Dehaut (2014),	yes

						Kouakou-Kouame (2020)
	3-methyl-butanal (isovaleraldehyde)	Malty, almond	oxidative cleavage of lipids, amino acid catabolism (L-Leucine)	1.1	African locust bean, anchovies	Ouoba (2015) Dehaut (2014)
Esters	Benzeneacetic acid methyl ester					
	Ethyl acetate					
	Acetic acid methyl ester					yes
	Methyl isovalerate - ester					
	Butanoic acid 2-methyl- ester	Floral and fruity notes	esters comes from chemical reactions between microbial acidic and alcoholic metabolites		Bambara , Adjuevan	Akanni (2018), Kouakou-Kouame (2020)
	Butanoic acid methyl ester					yes
	Acetic acid butyl ester					yes
	Butanoic acid butyl ester					yes
Ketones	Acetoin	butter-like aroma	Product of carbohydrates metabolism	800	bambara	Akanni (2018),
	2-Decanone	orange like floral*				
	2-Octanone	butter-like aroma*			Fruit wine	Feng (2015) yes
	Benzyl methyl ketone					
	Acetone		products of lipids and or amino acids degradation		ALBS	Ouoba (2015)
	Acetophenone Results	sweet and floral odors, almond			Adjuevan	Kouakou-Kouame (2020)
	2,3-Butanedione	toffee, butter, candy	Fermentation of sugars		anchovies, mushrooms	Dehaut (2014), Aisala (2019) yes
	Bicyclo[3.1.1]hept-3-en-2-one, 4,6,6-trimethyl	Camphoreous *				yes
Amides	Butanamide					
	Acetamide				Cocoa beans	Kuhnert (2020)
	N-(3-Methylbutyl) acetamide		reactions of peptides with Acids		Wine	Schueuermann (2016)
	Propanamide					
	2-methyl-propanamide,					
	3-methyl-butanamide					
Others	Phenol	sickening, sweet and irritating	heat-generated compounds		Milk	Dursun (2017)
	1-Butanamine					
	2-pentyl-Furan	fruity green earthy beany vegetable *			Baijiu	Zhang (2021) yes

Carbon disulfide	pungent and sulfury odors	oxidation of the reducing sulfur compounds	bambara, Milk	Akanni (2018), Dursun (2017)	yes
1,3-dimethyl-benzene,					yes
Toluene					yes
2-pentyl-thiophene,	Wine	thermal degradation of lipid oxidation products with amino acids and reducing sugars, cysteine	Model media	Song (2019)	
Oxime methoxy-phenyl		reaction aldehydes or ketones with a nitrogen-containing reducing agent in a weakly acidic medium	Milk	Dursun (2017)	yes
Ethylbenzene	gasoline				yes
3-Acetyl-1H-pyrroline	popcorn, basmati rice		Tomato pepper pomace, mushrooms	Aisala (2019)	
Glycerin					yes
Trimethyl-oxazole	Nutty, sweet, green				
Indole	manure, burnt		Adjuevan	Kouakou-Kouame (2020)	yes
2-Piperidinone	ammoniacal, pepper-like		mushrooms	Aisala (2019)	
D-Limonene	orange*				

*<http://www.thegoodscentscompany.com/data/rw1007681.html>

Table 1. Most frequent VOCs produced during fermented ALB production