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Full Length Research Paper

Use of starter cultures of *Bacillus* and *Staphylococcus* in the controlled fermentation of *Lanhouin*, a traditional fish-based condiment from West Africa

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Strains of *Bacillus subtilis*, *Bacillus licheniformis* and *Staphylococcus xylosus* previously isolated during the spontaneous fermentation of *Lanhouin* were tested singly and in combination for their ability to ferment pasteurized fish flesh to produce a new type of *Lanhouin*, a traditional condiment product. The growth of viable cells during the fermentation was monitored according to standard microbiological methods. Changes in pH, moisture and total volatile nitrogen (TVN) were determined according to Ababouch (1995) and AOAC (1995). The results revealed that both *B. subtilis* and *B. licheniformis* used as single or mixed cultures showed similar growth potential with a bacterial load of 8.0 Log (cfu/g) and pH value ranging between 6.8 and 6.9 after 72 h of fermentation. *S. xylosus* used as single starter culture also showed a good growth potential with however, a higher level of microbial load, 12.5 Log cfu/g and a pH value of 7.2. *Lanhouin* produced with the mixture culture of *B. subtilis*, *B. licheniformis* and *S. xylosus* gave a sauce with a desirable taste and aroma preferred by 100% of the panellists.

Key words: Fish, starter, inoculation, *Bacillus licheniformis*, *Bacillus subtilis*, *Staphylococcus xylosus*.

INTRODUCTION

In Benin, the artisanal fishery sub-sector provides about 94% of the domestic fish supply and accounts for 31% of national intake of animal protein (Anon, 2005; FAO, 2004). Artisanal fishery catches are sold fresh and mainly processed according to the storage conditions and taste of consumers (FAO, 2004). In fact, in Benin, like other African countries, cold storage facilities are limited at the landing sites and in the markets. In addition, high ambient temperature coupled with unsanitary handling conditions cause fish spoilage within 12 h (Gram, 2003; Gram and Dalgaard, 2002). Consequently, a large part of the artisanal catch that cannot be marketed immediately is dried, smoked or fermented for preservation. In 2002, the proportion of cured fish was higher in Africa (16%) and Asia (11%) compared with other continents (FAO, 2004). Thus, the improvement of the quality of fish through technological advances is important to reduce post harvest losses so as to utilize the country's resources to their full potential. Traditionally in Benin, many commodities (maize, sorghum, millet and fish) are processed by the cottage industries into fermented foods, which constitute the most important part of the staple foods, beverage, weaning foods and condiments consumed in the country (Azokpota et al., 2006; Anihouvi et al., 2005; Hounhouigan et al., 1993; Michodjèhoun-Mestres et al., 2005).

Lanhouin, a traditional salted and fermented fish product is mostly produced in the coastal regions and consumed by all the classes of the population in the south of Benin. The product which is similar in color, taste

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and smell to that of other fermented fish products such as momone which retain their original texture is widely used as taste enhancer in many traditional dishes and low coast meat substitute (Anihouvi et al., 2005; Abbey et al., 1994; Essuman, 1992). For *Lanhouin* processing, the fish is scaled, gutted and sometimes cut into pieces and then left overnight at ambient temperature in fly free enclosure. The next day, the seemingly spoiled fish is washed. Dry salt is rubbed into gills, the belly cavity and on the surface. After this first salting, the fish is arranged in a basket, a can or a hole, covered with old cement paper bag and old clothes and allowed to ferment for 3 to 8 days at room temperature (28 to 30°C) before being removed, washed slightly and sun dried (Anihouvi et al., 2005).

Lanhouin is produced by spontaneous and largely uncontrolled fermentation. Like other Beninese fermented products, it is currently produced largely on a traditional small-scale basis under highly variable conditions (Anihouvi et al., 2005; Essuman, 1992). The quality of the product is unpredictable and shelf-life is short. With increasing urbanization and demand of high quality traditional products, there is a need for controlled fermentation process and to minimize the variation in product quality encountered during the spontaneous fermentation at cottage industry level. In this respect, the use of starter cultures to promote *Lanhouin* processing was necessary. The use of starter cultures to promote traditional processing has been suggested by various workers (Anihouvi, 2006; Essuman, 1992; Holzapfel, 1997; Steinkraus, 2004; Terlabie et al., 2006). Various microorganisms belonging to the genera *Bacillus*, *Staphylococcus*, *Micrococcus*, *Streptococcus*, *Corynebacterium, Pseudomonas*, *Achromobacter* and *Alcaligenes* were isolated during a previous natural fermentation study, and the predominant ones were identified at the species level (Anihouvi et al., 2007). Among *Bacillus* spp. and *Staphylococcus* spp., *Bacillus subtilis* and *Bacillus licheniformis*, and *Staphylococcus xylosus* were the predominant species isolated and identified (Anihouvi et al., 2007). Some biochemical changes occurring during this type of fermentation have also been investigated and the results showed that the moisture and protein contents decreased while pH, free fatty acid (FFA) and total volatile nitrogen (TVN) increased with fermentation time (Anihouvi et al., 2009). In contrast, there was no linear relationship between the histamine content and fermentation time since the histamine values did not increase continuously as fermentation progressed (Anihouvi et al., 2009). The present study aims to investigate the ability of the predominant organisms previously isolated during the natural fermentation to be used as starter cultures for the controlled fermentation of *Lanhouin* and to identify the role of this individual micro-organism during the previous natural fermentation.

MATERIALS AND METHODS

Materials

Fish and salt

Fresh cassava fish (*Pseudotolithus* sp.) was purchased at Cotonou seaport (Bénin). The fish was placed in an ice-chest filled with ice and transported to the laboratory. The fish was selected on the basis of its common use to produce traditional *Lanhouin* (Anihouvi et al., 2005). Imported salt used for fish curing was obtained from the local market.

Strains of microorganisms

Cultures of *B. subtilis* (Bs), *B. licheniformis* (Bl), and *S. xylosus* (Sx) originally isolated and identified during a previous study and representing predominant micro-organisms associated with spontaneous fermentation of *Lanhouin* (Anihouvi et al., 2007) were used as starter to ferment fish meat. The strains were preserved as frozen glycerol suspensions and stored at -20°C.

Methods

Preparation of fish

Fresh fish (*Pseudotolithus* sp.) was purchased at the seaport and transported in an ice chest filled with ice to the laboratory. The fish was washed, scaled, gutted, beheaded and washed twice before filleting. The fish flesh was then separated from the skin and bones and ground using a blender (Waring Commercial Blender 35B64, USA). The ground fish flesh was then collected into a baker and pasteurized at 80°C for 30 min using an electric steam sterilizer (Systec, 3870 MLV, NL), followed by rapid cooling to about 30°C by placing the baker in a bath filled with water and ice. During pasteurization, the vane of the sterilizer was left open to avoid an increase of pressure.

Preparation of inocula

The stock cultures were subcultured in Nutrient agar (NA, Oxoid CM3, Basingstone, Hampshire, England) and incubated for 24 h at 35 and 30°C for *Bacillus* species and *S. xylosus*, respectively. After successive subculturing in nutrient broth (Oxoid CM0001, Basingstone, Hampshire, England), each culture was washed in 10 ml sterile 0.9% NaCl solution and diluted to obtained concentrations of 10^6 cells /ml. Cells concentrations were checked as viable count in NA (Oxoid CM3, Basingstoke, Hampshire, England) and by using a Thomas Counting Chamber.

Fermentation trials

For the fermentation, 75 g of sterilized salt were added to 1500 g of pasteurized fish flesh and the mixture inoculated with 15 ml of inoculum, and then homogenised in aseptic conditions (this gave approximately a concentration of 10^6 cells/g of fish meat). For the mixed starter cultures, the suspensions of different cultures were mixed equally and 15 ml of the final mixed culture inoculated into 1500 g of pasteurized fish flesh. The ingredients were mixed by hand under aseptic conditions and the inoculated fish mixtures were packed in batch of 200 g into 250 ml sterilized conical flask

covered with cotton wool. The inoculated fish mixtures were then incubated at 35°C for 72 h. Samples were taken at 0, 12, 24, 36, 48, 60 and 72 h of fermentation for microbiological and chemical analyses. In total, seven samples, each representing one fermentation time, were fermented at two different occasions for each single or combined starter culture. Similarly, one non-inoculated sample was used as control at each fermentation time.

Microbiological analyses

For all samples, 10 g was introduced aseptically in a sterile stomacher bag (Seward Medical, London, UK) and macerated for 2 min in 90 ml of sterile diluent [0.1% peptone (Oxoid L 37, Basingstoke, Hampshire, England), 0.8% sodium chloride (NaCl) with pH adjusted to 7.2] using a stomacher (Lab Blender, Model 400, Seward Medical, London, England). One (1) ml of the suspension was serially diluted and used for enumeration of micro-organisms. *Bacillus* species and *S. xylosus* were enumerated using Tryptone soya agar (TSA, Oxoid CM 75, Basingstoke, Hampshire, England) and Mannitol salt agar (MSA, Oxoid CM 85, Basingstoke, Hampshire, England) respectively. TSA plates were incubated for 48 h at 35°C whereas plates for *Staphylococcus* were incubated at 30°C for 72 h. Plates having between 30 and 300 colonies forming units (cfu) per gram were counted and the results were expressed as Log cfu per g wet weight of sample.

Physico-chemical analyses

pH of fermenting fish mixtures was measured with a pH-meter (Hanna Instrument HI 9318) on a mixture of 20 g of blended fish meat and 80 ml of distilled water. TVN was determined according to Ababouch (1995). Ten (10) g of sample was blended and added to 90 ml 6% perchloryde acid. The mixture was then homogenised for 2 min, filtered and 50 ml of the filtrate was transferred in a distilling flask of macro Kjeldahl for distillation. The distillate collected in 3% boric acid was titrated with 0.05 N HCL. Moisture was determined using the method of AOAC (1995). In the procedure, about 5 g of blended fish muscle were weighed into a metal dish containing about 20 g of treated sand. The sample was mixed with the sand using the glass rod to achieve as homogeneous a mixture as possible. The dish, its content including the rod were dried to constant weigh at 103±2°C.

Sensory analysis

The different sauces prepared with *Lanhouin* obtained with Bs+Bl, Sx+Bl, Sx+Bs and Sx+Bs+Bl as mixed starter cultures for the fermentation, were evaluated by 21 untrained judges but familiar with *Lanhouin*. The sauces were presented to the panellists in a random order using 3–degit random number codes and were asked to indicate their preference in a hedonic preference test (Watts et al., 1991). Attributes (aroma and taste) were assessed using slimy leafy vegetable (*Corchorus olitorius* L) sauces prepared as follows: 30 g of *Lanhouin*, 50 g leafy vegetable and 200 ml of tap water. The mixture was cooked for 30 min using a heating plate.

Statistical analyses

Statistical package for social science (SPSS version 10) was used. Data analyses involved one-way analysis of variance (ANOVA). Means difference were determined using Dunnett and Student-Newman-keuls tests. Significant difference was established at 5%.

RESULTS AND DISCUSSION

Changes in viable cells during the fermentation

The growth of various bacteria species inoculated in single during the fermentation of cassava fish flesh is shown in Figure 1a. A gradual increase of *S. xylosus* was noted during the first 60 h of fermentation, followed by a slight decrease and a final microbial load of 13.4 Log (cfu/g) after 72 h of fermentation. This high ability of staphylococci to ferment meat product such as sausage was observed by Nychas and Arkoudelos (1990). The *Bacillus* starter cultures showed similar trend, with however, more limited growth and microbial counts ranging from 5.9 to 8.0 Log (cfu/g) for *B. subtilis* and 5.8 to 7.9 for *B. licheniformis* after 72 h of fermentation, respectively; while no growth was observed in the control sample. The Figures 1b, 1c 1d and 1e showed the dynamic of growth of different cultures used in combination; in general, a gradual increase of individual starter culture was also noticed. However, the Figures 1b and 1c showed that when one of *Bacillus* species under study is used individually in association with *S. xylosus*, its number decreased at 48 h of fermentation. In contrast, this decrease was not observed when the two species of *Bacillus* are used in combination with *S. xylosus* (Figure 1e). In addition, the period of decrease fit with the peak of growth of *S. xylosus* indicating that *S. xylosus* has a higher ability to ferment the fish mixture. Thus, the decrease observed in *Bacillus* micro-population at 48 h of fermentation could be due to the nutritional competition between the different microorganisms introduced in the fish flesh (Elizabeth and Fitzgeralda, 1999); this could also be due to the level of organic acid produced in the medium by *S. xylosus* during the fermentation. According to Annalisa et al. (2007) and Gonzalez and Diez (2002) *S. xylosus* is mostly used during the processing of fermented sausages for its ability to produce organic acids. From this investigation, it appeared that starter cultures of *B. subtilis*, *B. licheniformis* and *S. xylosus* can be used to control the fermentation. However, the ability of test organisms to ferment the fish mixture varied significantly ($p<0.05$) between *Bacillus* species and *S. xylosus*. On the safety aspect, both *B. subtilis* and *B. licheniformis* have been identified as main organisms responsible for the alkaline fermentation of African locust bean into various traditional products in West Africa (Azokpota et al., 2006; Steinkraus, 1991). *S. xylosus* has been rarely associated with human or animal infections (Kloos and Schleifer, 1986) and *S. xylosus* is mostly used as starter culture in the fermentation of meat products (Metz, 1993; Nychas and Arkoudelos, 1990; Sondergaard and Stahnke, 2002).

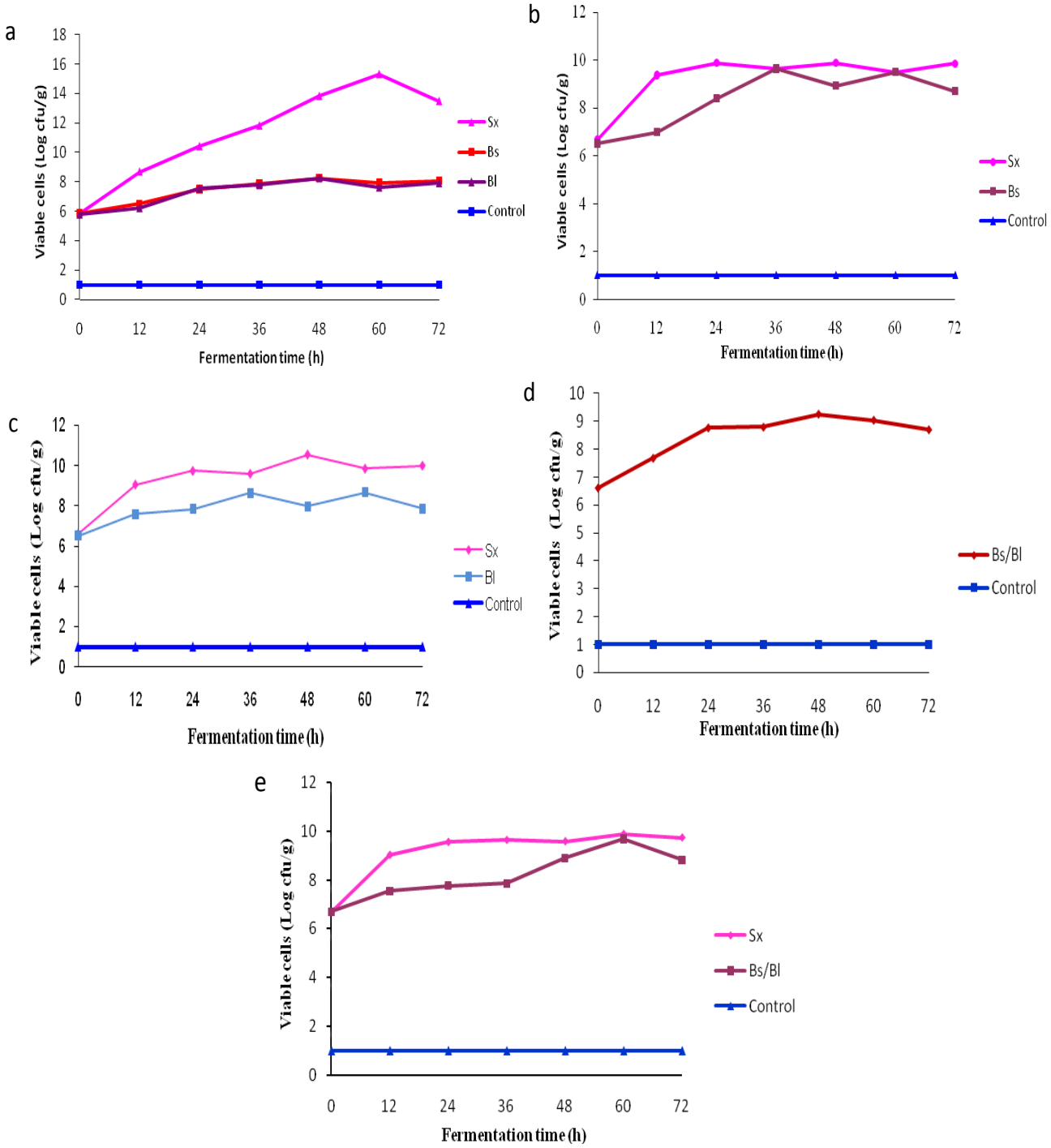

Figure 1. Changes in viable cell counts during *Lanhouin* production with single (a) and mixed (b, c, d, e) starter cultures of *Bacillus* spp. and *S. xylosus*. Bs, *B. subtilis*; Bl, *B. licheniformis*; Sx, *S. xylosus*; a, single starter cultures of *B. subtilis*, *B. licheniformis* and *S. xylosus*; b, mixed starter culture of Sx + Bs; c, mixed starter culture of Sx + Bl; d, mixed starter culture of Bs + Bl; e, mixed starter culture of Sx+ Bs + Bl; control: non inoculated sample.

Changes in pH

For all fermentations, the pH of the fermenting fish mix increased significantly ($p<0.05$) as the fermentation progressed (Figures 2a and b); however, the three species of microorganisms used as mono culture showed

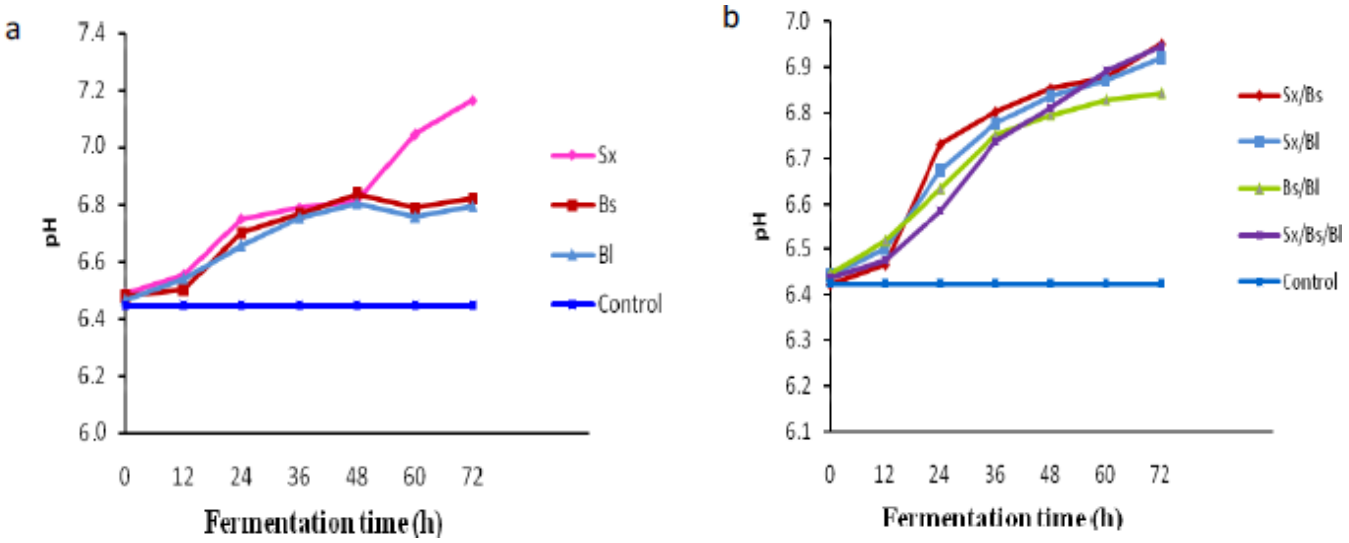

Figure 2. Changes in pH during *Lanhouin* production with single (a) and mixed (b) starter cultures of *Bacillus* sp. and *S. xylosus*. Bs, *B. subtilis*; Bl, *B. licheniformis*; Sx, *S. xylosus*; Sx/Bs, mixed starter culture of Sx+ Bs; Sx/Bl, mixed starter culture of Sx + Bl; Bs/Bl, mixed starter culture of Bs + Bl; Sx/Bs/Bl, mixed starter culture of Sx + Bs + Bl; Control, non inoculated sample.

different levels of pH, ranging from the initial value of 6.3 to 7.2 and 6.8 after 72 h of fermentation for *S. xylosus*, *B.subtilis* and *B. licheniformis*, respectively (Figure 2a). This showed that the test organisms used as single inoculum developed different ability to increase the pH values, with *S. xylosus* giving the most alkaline product, whereas the pH of the non-inoculated samples (control) remained the same throughout fermentation. In addition, when S. xylosus is used in combination with one or the two species of *Bacillus*, the fermenting fish showed the higher value of pH, with a mean final pH values of 7.0 for the mixed inoculum of S*. xylosus, B. subtilis* and *B. licheniformis*, and 6.8 for the mixed inoculum of the two species of *Bacillus* (Figure 2b). From these observations, it appeared that the presence of *S. xylosus* in the two-membered starter culture increased the alkalinity of the fish mixture compared to *B. subtilis* and *B. licheniformis* used as mono inoculum, and *B. subtilis* and *B. licheniformis* as mixed inoculum. In the same way, *S. xylosus* in the three-membered starter culture increased the alkalinity of the final product. This suggested that the test organisms used, particularly *S. xylosus* had an important proteolytic activity, leading to the increase of pH during the spontaneous fermentation of fish. The rise in pH was possibly due to the formation of nitrogenous basic compounds as the result of microbial proteolytic activity of the inoculated organisms (Kiers et al., 2000; Steinkraus, 1985) since the fish flesh was pasteurized before inoculation. A similar trend of pH during the spontaneous fermentation of fish has been reported by various workers (Anihouvi et al., 2007; Achinewhu and Oboh, 2002; Abbey et al., 1994; Omafuvbe et al., 2002; Sakar and Tamang, 1995; Subasinghe et al., 1990). In addition, the proteolytic activity of *S. xylosus* has been reported by Miralles et al. (1996) and Talon et al. (2002).

Changes in moisture

Figure 3 shows the variation in moisture content during the controlled fermentation using various inocula. In general, the moisture content of the fermenting fish samples including the control samples (non-inoculated samples) increased as fermentation progressed and reached after 72 h of fermentation mean values of 74% for the inoculated samples against 71.5% for the control (Figures 3a and b). No significant difference was noted ($p > 0.05$) in moisture contents within the inoculated samples but a significant difference ($p < 0.05$) was observed between the moisture content of inoculated samples and the control. The increase in moisture contents during the fermentation could be attributed to the combined effect of salt, heat treatment and the proteolytic activity of inoculated micro-organisms. According to Horner (1997), the combined effect of salt and heat treatment may impact the retention capacity of the fish meat leading to the release of water. In addition, the proteolytic activity of inoculated organisms could also contribute to the release of water through the hydrolysis of peptides. Therefore, the significant difference observed between the moisture contents of the inoculated samples and the control could be attributed to the proteolytic activity of the inoculated microorganisms. Similar increase in moisture content has been reported by Twiddy et al. (1987) during the

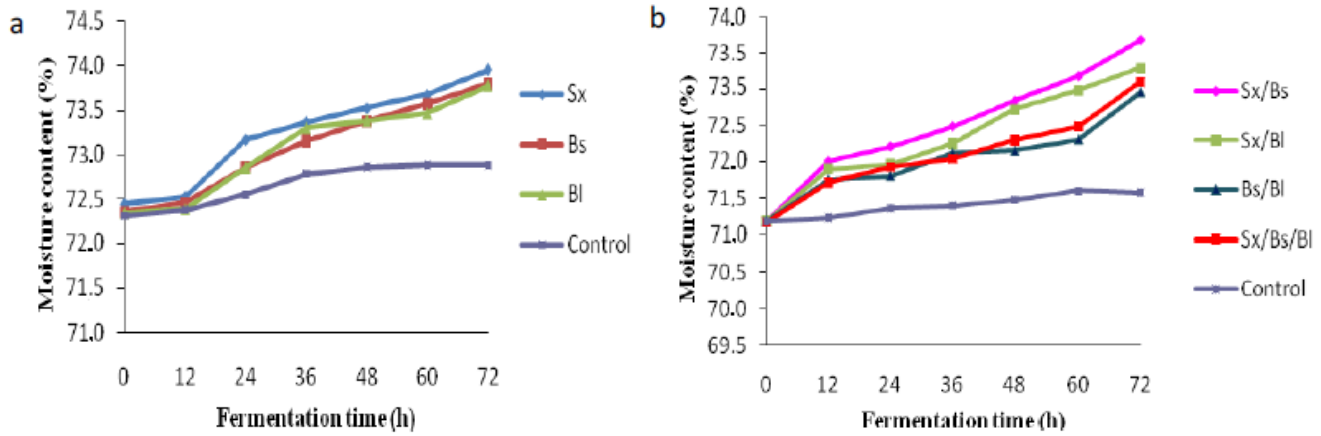

Figure 3. Changes in moisture during *Lanhouin* production with single (a) and mixed (b) starter cultures of *Bacillus* spp. and *S. xylosus*. Bs, *B. subtilis*; Bl, *B. licheniformis*; Sx, *S. xylosus*; Sx/Bs, mixed starter culture of Sx+ Bs; Sx/Bl, mixed starter culture of Sx + Bl; Bs/Bl, mixed starter culture of Bs + Bl; Sx/Bs/Bl, mixed starter culture of Sx + Bs + Bl; Control, non inoculated sample.

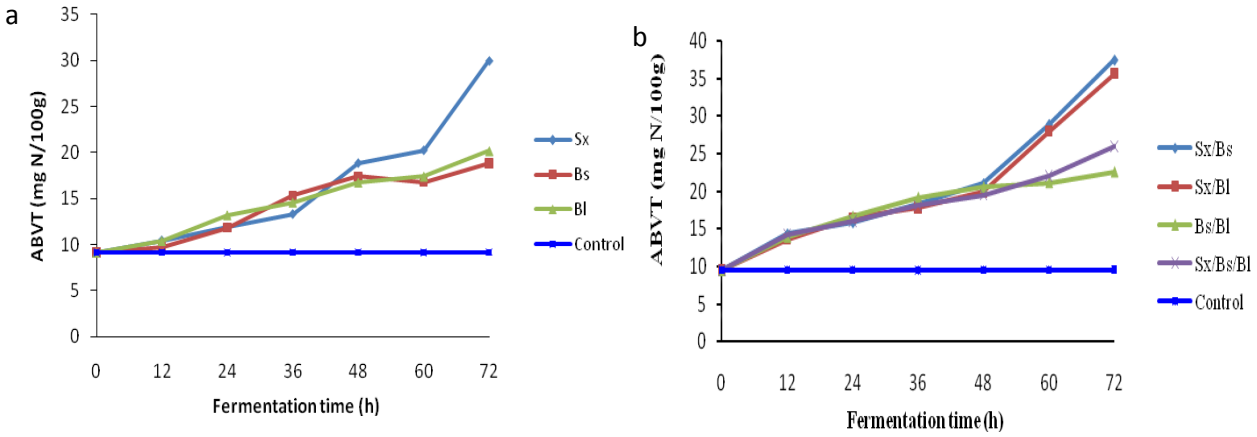

Figure 4. Changes in TVN content during *Lanhouin* production with single (a) and mixed (b) starter cultures of *Bacillus* spp. and *S. xylosus*. Bs, *B. subtilis*; Bl, *B. licheniformis*; Sx, *S. xylosus*; Sx/Bs, mixed starter culture of Sx+ Bs; Sx/Bl, mixed starter culture of Sx + Bl; Bs/Bl, mixed starter culture of Bs + Bl; Sx/Bs/Bl, mixed starter culture of Sx + Bs + Bl; Control, non inoculated sample.

production of lactic fermented fish product.

Changes in total volatile nitrogen

An increase in TVN was observed over the fermentation period for all the samples obtained from all the inocula used, except the control samples (Figure 4). When the test organisms are used as single inocula, the TVN values increased from the initial mean value of 9.1 mg N/100 g and reached after 72 h a final values of 30.0 mg N/100 g and 19.5 mg N/100 g for samples inoculated with cultures of *S. xylosus* and *Bacillus* species, respectively; while the TVN content of the control remains stable (Figure 4a). An increase in TVN was also observed when the test microorganisms are used as mixed inocula, but the highest levels of TVN were obtained in the combinations including *S. xylosus* (Figure 4b). The increase in TVN resulted from the formation of nitrogenous basic compounds, such as ammonia, due to the degradation of protein through microbial activity of inoculated organisms. Similar increase in TVN has been reported by Yongjin et al. (2007) during the controlled fermentation of fish meat for the production of fish sausage. These results showed that both *Bacillus* species used and mainly *S. xylosus* could play a significant role in aroma development and textural changes during the spontaneous fermentation of fish for *Lanhouin* production. In this respect,

Table 1. Mean sensory score for sauces prepared with the inoculated Lanhouin samples.

Inocula used	Sensory attributes					
	Aroma			Taste		
	48 h	60 h	72 h	48 h	60 h	72 h
Bs/Bl	3.20 ± 1.19[1]a	3.25 ± 0.85a	3.50 ± 0.94a	3.35 ± 1.19a	3.90 ± 0.85a	4.05 ± 0.60a
Sx/Bl	2.70 ± 0.86a	3.30 ± 0.97a	3.35 ± 1.03a	3.50 ± 1.00a	3.90 ± 0.85a	3.30 ± 1.03b
Sx/Bs	3.45 ± 1.05a	3.20 ± 0.83a	3.25 ± 1.06a	4.05 ± 0.88a	4,00 ± 0.97a	3.65 ± 0.87ab
Sx/Bs/Bl	3.05 ± 0.82a	3.30 ± 1.03a	3.65 ± 0.98a	3.65 ± 1.03a	4,00 ± 1.07a	4.15 ± 0.67a

a and b, Means with different letters in a row are significantly different (p <0.05). Bs, *B. subtilis*; Bl, *B. licheniformis*; Sx, *S. xylosus*; Sx/Bs, mixed starter culture of Sx+ Bs; Sx/Bl, mixed starter culture of Sx + Bl; Bs/Bl, mixed starter culture of Bs + Bl; Sx/Bs/Bl, mixed starter culture of Sx + Bs + Bl; Control, non inoculated sample.

Staphylococcus species, particularly *S*. *xylosus*, *Staphylococcus carnosus* and *Staphylococcus equorum* were used in a comparative study for their ability to produce aroma (Sondergaard and Stahnke, 2002). The level of TVN in fish and fish products is mostly used as spoilage indicator through bacterial activity (Gram, 2003). According to Silva et al. (1998), TVN level below 20 to 36 mg N/kg indicate that the fish is fresh, whereas the fish would be rejected for human consumption when the TVN level exceeds approximately 500 mg N/kg. The TVN levels recorded on our samples were within acceptable limits.

Sensory evaluation

The Table 1 shows the results of sensory evaluation of *Lanhouin* fermented with the mixed starter cultures of *Bacillus* species and *S. xylosus*. From these results, it appeared that all the sauces prepared with *Lanhouin* samples fermented for 48, 60 and 72 h using the combination of two species of *Bacillus*, the combination of *S. xylosus* and one of the two species of *Bacillus* and the combination of three species under study do not differ significantly (p>0.05) in terms of aroma. In addition, no significant difference was noted (p>0.05) between the sauces prepared with *Lanhouin* produced with the same inocula and fermented for 48 and 60 h in terms of taste; in return, the samples inoculated with the combinations of Sx+Bs and Sx+Bl were judged different after 72 h of fermentation. Only *Lanhouin* samples produced with the mixture culture of three strains (*B. subtilis* + *B. licheniformis* + *S. xylosus*) gave a sauce with a desirable taste and aroma preferred by 100% of the panellists.

Conclusion

From this preliminary investigation, it appeared that the strains of *B. subtilis*, *B. licheniformis* and *S. xylosus* isolated from a previous spontaneous fermentation can be used as mixed starter cultures to control the fermentation. However, only the sauce prepared with *Lanhouin* fermented with the mixed starter cultures of three strains under study gave the most desirable sauce by the panellists. *Bacillus* species are mostly considered as the main organisms responsible for alkaline fermentation of many traditional fermented foods including fish products. The present study revealed that coagulase negative *Staphylococcus* species such as *S. xylosus* should be considered as a potential technological microorganism in alkaline fermentation, mainly in fish fermentation area. Further investigations need to be performed for a better understanding of the controlled fermentation of fish. This could provide comprehensive knowledge on the processing which will facilitate the development of a modern food condiment along the lines of the “bouillon cube”.

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