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STUDY OF LACTIC ACID BACTERIA AND *ENTEROBACTERIACEAE* COUNTS AND FATTY ACIDS COMPOSITION IN A FERMENTED PRODUCT

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SUMMARY

This study was conducted to study the characteristics of a fermented product (FP). An evaluation of measurements of consistency and repeatability on microbiological analyses, including lactic acid bacteria (LAB) and *Enterobacteriaceae* counts and fatty aids profiles was performed. Physico-chemical properties (pH, dry matter and ash, crude protein, crude fat and crude fibre) of FP were ascertained. The fermentation process was repeated three times under the same running conditions and using the same amounts of raw materials. The results indicated that the product obtained from the fermentation process increased the LAB population and decreased the *Enterobacteriaceae* populations after the fermentation process. The presence of essential fatty acids (linoleic acid 24%, linolenic acid 1.1%, Eicosapentaenoic acid 3.6%, Docosapentaenoic acid 0.7%, and Docosahexaenoic acid 2.0%) and lime-flavored aroma represents added value attributes to the product and it might have potential as an animal feed additive. Chemical determination showed a net decrease in pH to around 4.5. The dry matter, crude fibre, crude protein, crude fat and ash contents of the three batches of FP were similar. These results indicated that the quality of FP was consistent with repeatable preparation process.

Keywords: Fermented product, fermentation, lactic acid bacteria, *Enterobacteriaceae*, fatty acid profile

INTRODUCTION

Fermentation is one of the oldest technologies used for food and feed preservation. Over the centuries, the fermentation process has been evolved and refined extensively to produce a diversified range of products (Prajapati and Nair, 2003). It is possible to obtain a large variety of fermented products by selecting different types and compositions of raw materials, starter cultures and fermentation conditions (Hansen, 2002). Generally, fermented products contain high numbers of lactic acid bacteria, a low pH and a high concentration of lactic acid. The physical, chemical and biological characteristics have been modified by the activity of microorganisms. Microorganisms, by virtue of their metabolic activities, contribute to the development of characteristic properties such as taste, aroma, texture, visual appearance, shelf life and safety of the product (Holzapfel, 2002). The actions of microorganisms during the preparation of cultured foods play an important role in improving the safety of feeds by removing their natural toxic components, or by preventing the growth of disease-causing microbes (e.g. *Enterobacteriaceae*). Fermentation with LAB cultures has been shown to be able to improve the quality, availability and digestibility of dietary nutrients. LAB improves protein digestibility and micronutrient bioavailability of fermented feed through the biosynthesis of vitamins and essential amino acids. Thus, they play an essential role in food and feed fermentation and are well known for their acidification of the product (Hammes *et al.*, 1990).

During the early ages of civilisation, fermented products were mainly used for human consumption. The beneficial LAB present in the products is well documented for its antimicrobial compounds such as bacteriocin, as a biopreservative for food products (Schnürer and Magnusson, 2005). Products derived from LAB fermentation have the potential to prevent various human diseases (Minamiyama *et al.*, 2003). Recently, fermented liquid feed has been used as an animal diet. Fermented liquid feed has also shown promising results in enhancing the performance of animals (Mikkelsen and Jensen, 1998; Demecková *et al.*, 2002). However, fermented liquid feed is tedious to prepare and apply. The solid form FP investigated in this study was designed to overcome these limitations. Most of the studies on fermentation had been done on animal and agricultural by-products (Kamra and Srivastava, 1994; Joshi and Sandhu, 1996; El Jalil *et al.*, 2001). Up to date, none of the fermentation studies done on agricultural by- products involved the addition of marine fish in order to enhance the nutritive value of the final product. In the present study, fish

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was used to increase the levels of polyunsaturated fatty acids (PUFA) as PUFA have been used successfully to manipulate and increase the n-3 levels in animal products for livestock production (Milinsk *et al.*, 2003). Thus, the objectives of this study were to evaluate the chemical composition and nutritive value of the product after the process of fermentation.

MATERIALS AND METHODS

Preparation of fermented product (FP)

The fermentation process was performed according to the method as described by Loh *et al.* (2003a). The raw materials consisted of 9% (wt/wt) lime, 1% (wt/vol) molasses, 53.5% (wt/wt) rice bran, 35% (wt/wt) fish (*Rastrelliger kanagurta*), 1% (wt/vol) vinegar, and 0.5% (wt/wt) starter cultures. The starter culture was a combination of *Lactobacillus sp.* with a population of 3.56×10^4. The starter culture was obtained from Jia Yi Nutritional Sdn. Bhd (Klang, Malaysia). Lime was blended with molasses and the mixture was then fermented at ambient temperature (28±2°C) for five days under aerobic conditions. The mixture was then poured into a closed 50L solid fermenter together with rice bran, raw fish, vinegar and starter culture. The fermenting mixture was mixed hourly in order to achieve a homogenous mixing. The fermentation process was carried out for seven days at 35-38°C. Fermentation was considered complete after seven days. The completed FP was allowed to cool to room temperature prior to sampling. The final product was a dry-brownish mash with a distinctive lime-flavoured aroma. The FP was stored in a vacuum-sealed black plastic bag until use.

Chemical and microbiological analyses

The pH of the FP was measured with a Mettler-Toledo pH meter (Mettler-Toledo LTD., England) according to the method as described by Loh *et al.* (2003b). Ten percent (wt/vol) of the sample was mixed homogenously with deionised distilled water. Dry matter was determined by oven drying at 103°C to a constant weight. Ash was determined by ignition in a muffle furnace at 600°C. The crude protein (micro Kjeldahl method), crude fibre and crude fat (Soxhlet method with petroleum ether as solvent) were analysed as per AOAC (1990) procedure. The microbiological analyses were done according to the method as described by Loh *et al.* (2003a;b). Enumerations of total lactobacilli were performed on MRS-agar (*Lactobacillus*-Agar DE Man, ROGOSA and SHAPE) (Merck). The plates were incubated in an anaerobic jar at 30°C for 48 hours. *Enterobacteriaceae* were counted on EMB-agar (Eosin-methylene-blue Lactose Sucrose Agar) (Merck) and incubated aerobically for 24 hours at 37°C. Numbers of colony-forming unit (cfu) were expressed as $\log_{10}$ cfu/g. All samples were performed in triplicate.

Determination of fatty acids profiles

The total lipids were extracted from the product using a modified Folch *et al.* (1957) method as described by Rajion *et al.* (2001) using chloroform-methanol 2:1 (vol/vol) solvent system. Transmethylation was carried out using 14% methanolic boron trifluoride (Sigma Chemical Co., St. Louis, Missouri, USA). The derived fatty acid methyl esters (FAME) were separated by gas chromatography using a 5890 Hewlett-Packard Gas-Liquid Chromatograph (Hewlett-Packard, Avondale, PA) fitted with a flame ionisation detector (FID) and a Supelco SP-2330 fused silica capillary Column (30m, 0.25mm ID, 0.20μm film thickness) (Supelco, Inc., Bellefonte, PA). The column temperature was programmed at 7.2°C/min from 100 to 190°C. The injector and detector were programmed at 220°C and 220°C, respectively. High purity nitrogen was the carrier gas at a flow rate of 40ml/min. An internal standardisation method was used to quantify the various fatty acids in the FP, where a known concentration of heneicosanoic acid was added to each sample prior to transmethylation. Peak areas were determined by the HP-3993A Integrator (Hewlett-Packard, Avondale, PA). The identification of the peak was made by comparison of equivalent chain lengths (ELC) with those of authentic fatty acid methyl esters (Sigma Chemical Co., St. Louis, Missouri, USA).

Statistical analysis

Results were expressed as mean ± standard error of the mean. Total lactobacilli and *Enterobacteriaceae* counts were expressed as $\log_{10}$ cfu/g. The entire colony counts, pH value, fatty acid profile, crude protein, crude fibre, crude fat, dry matter were recorded and analysed by one-way ANOVA using SAS (SAS®, 1991). Duncan's Multiple Range Test was used to compare the differences of mean and the level of significance was $P < 0.05$.

RESULTS AND DISCUSSION

Microbiological characteristics of FP

The fermentation process performed in this study demonstrated reduction of harmful bacteria such as *Enterobacteriaceae* in the end product compared to the raw materials (Table 1). In contrast, the population of LAB increased in the end product as compared to the raw materials. The results obtained in this study were consistent with the findings of other reports (Mikkelsen and Jensen, 2000; van Winsen *et al.*, 2002; Demecková *et al.*, 2002; Loh *et al.*, 2003a; b; Heres *et al.*, 2004). The use of potentially beneficial bacteria such as LAB has been demonstrated to inhibit the *in-vitro* growth of many

Table 1: Total lactobacilli and *Enterobacteriaceae* counts in raw materials and finished FP

Microorganisms Log_{10}(cfu/g)	Fermentation time	Batch 1 (n=3)	Batch 2 (n=3)	Batch 3 (n=3)
Total Lactobacilli	Day 0	$4.28^{a,x} \pm 0.01$	$4.40^{b,x} \pm 0.01$	$4.37^{b,x} \pm 0.02$
	Day 12	$6.26^{a,y} \pm 0.03$	$6.25^{a,y} \pm 0.11$	$6.41^{a,y} \pm 0.02$
Enterobacteriaceae	Day 0	$3.87^{a,x} \pm 0.01$	$3.87^{a,x} \pm 0.02$	$3.83^{a,x} \pm 0.03$
	Day 12	$0.83^{a,y} \pm 0.16$	$0.87^{a,y} \pm 0.20$	$1.26^{a,y} \pm 0.41$

The results are presented as mean values ± SEM.
[a,b]Values with different superscripts within a row differ significantly at P<0.05 due to batch effects;
[x,y]Values with different superscripts within a column differ significantly at P<0.05 due to day effects

Table 2: Physico-chemical characteristics of the FP

Parameters	Batch 1 (n=3)	Batch 2 (n=3)	Batch 3 (n=3)
pH	$4.75^{a} \pm 0.02$	$4.21^{b} \pm 0.01$	$4.55^{c} \pm 0.01$
GE, Kcal/kg [ns]	4490.97 ± 32.20	4509.98 ± 8.70	4415.69 ± 156.00
(%, DM basis)			
DM [ns]	90.69 ± 0.83	92.65 ± 0.07	92.18 ± 0.23
Crude fiber [ns]	0.07 ± 0.01	0.06 ± 0.01	0.07 ± 0.01
Crude proteins [ns]	19.97 ± 0.10	19.00 ± 0.33	19.21 ± 0.22
Crude fat [ns]	0.11 ± 0.01	0.13 ± 0.01	0.13 ± 0.01
Ash [ns]	1.02 ± 0.01	0.99 ± 0.01	1.00 ± 0.02

The results are presented as mean values ± SEM.
Values with different superscripts within a row differ significantly at P<0.05;
[ns] No significant difference

enteric pathogens including *Salmonella typhimurium*, *Staphylococcus aureus*, *Escherichia coli*, *Clostridium perfringens* and *Clostridium difficile* (Lindgren *et al.*, 1990; de Vuyst and Vandamme, 1994; Forestier *et al.*, 2001). Furthermore, *in-vivo* study of feeding fermented feed improved the microbial balance in the GIT of animals (Urlings *et al.*, 1993; Mikkelsen and Jensen, 2000; van Winsen *et al.*, 2002; Demecková *et al.*, 2002; Loh *et al.*, 2003a,b; Heres *et al.*, 2004). It may be that the beneficial LAB present in the product produced a range of antimicrobial metabolites such as bacteriocins, ethanol, H_2O_2, and organic acids during fermentation (Ross *et al.*, 2002). Organic acids helped to reduce the pH value to a range of 4-5, which was detrimental for deleterious microbes (Østergaard *et al.*, 1998; Christine *et al.*, 1999; Beal *et al.*, 2002). This in turn, provided a suitable environment to promote the growth and sustenance of the lactic acid-producing microflora (Owen and Mendoza, 1985; Urlings *et al.*, 1993; van Winsen *et al.*, 2002; Loh *et al.*, 2003 a; b).

The undissociated, more hydrophobic form of the organic acids diffuses over the cell membrane. It dissociates inside the cell thus releasing H^+-ions that acidify the cytoplasm and reducing the intracellular pH. This in turn inhibits the active transport and a variety of metabolic functions (Doores, 1993; Ross *et al.*, 2002). In addition to the pH effect, the undissociated acid collapses the electro-chemical proton gradient, causing bacteriostasis and finally death of susceptible bacteria (Axelsson *et al.*, 1989 as cited by Schnürer and Magnusson, 2005) such as *Enterobacteriaceae* in the FP. The reduction of *Enterobacteriaceae* in the FP is important to product safety as it indicates a safe product and a successful fermentation process where hazardous microorganisms are inhibited. Thus, this product may be used safely in the animal feed industry for optimal growth performance and as a prophylaxis against infectious agents such as *Salmonella* spp.

Physico-chemical characteristics of FP

The dry matter (DM), crude fibre, ash, crude protein (CP), crude fat and gross energy (GE) contents and the fatty acids compositions were not significantly different (P>0.05) across batches except pH (P<0.05) which ranged from 4.21 to 4.75 (Table 2). These results indicate that the FP could be produced at a consistent quality under a reproducible fermentation process. Presence of beneficial long-chain fatty acids (PUFA) such as

Table 3: Fatty acid compositions of FP in different batches

Fatty Acids	Batch 1 Mean ± SEM (mg/100g)	%	Batch 2 Mean ± SEM (mg/100g)	%	Batch 3 Mean ± SEM (mg/100g)	%
C12:0	6.2 ± 1.1	0.1	5.9 ± 0.8	0.1	6.1 ± 1.4	0.1
C14:0	200.6 ± 13.1	2.9	193.7 ± 2.36	2.9	212.3 ± 22.6	2.9
C15:0	28.5 ± 9.0	0.4	26.9 ± 4.3	0.4	30.7 ± 11.6	0.4
C16:0	1444.8 ± 121.0	20.8	1393.5 ± 170.0	20.9	1525.0 ± 31.0	20.8
C16:1 n-9	270.6 ± 21.6	3.9	259.5 ± 34.8	3.9	287.8 ± 34.7	3.9
C17:0	30.2 ± 7.2	0.4	28.7 ± 5.8	0.4	32.3 ± 9.8	0.4
C17:1	40.7 ± 7.1	0.6	38.4 ± 5.6	0.6	43.1 ± 10.1	0.6
C18:0	294.3 ± 20.2	4.2	282.1 ± 37.5	4.2	311.7 ± 29.0	4.2
C18:1 n-9	2216.8 ± 387.0	31.9	2138.9 ± 237.0	32.0	2342.7 ± 231.0	31.9
C18:2 n-6	1671.4 ± 349.0	24.1	1613.2 ± 167.0	24.1	1764.2 ± 246.0	24.0
C18:3 n-3	73.3 ± 12.5	1.1	71.3 ± 5.6	1.1	76.7 ± 10.1	1.0
C20:0	49.7 ± 7.3	0.7	48.8 ± 6.1	0.7	52.2 ± 3.9	0.7
C20:1 n-9	35.2 ± 4.1	0.5	34.6 ± 4.2	0.5	37.0 ± 1.2	0.5
C20:4 n-6	86.3 ± 19.9	1.2	81.3 ± 15.8	1.2	91.9 ± 28.2	1.3
C22:0	24.1 ± 2.7	0.3	22.6 ± 3.4	0.3	25.4 ± 0.9	0.3
C24:0	36.5 ± 4.2	0.5	35.4 ± 4.9	0.5	41.2 ± 1.6	0.6
C20:5 n-3, EPA	246.9 ± 19.8	3.6	230.6 ± 22.4	3.5	261.8 ± 31.4	3.6
C22:5 n-3, DPA	46.4 ± 6.8	0.7	44.4 ± 8.2	0.7	50.1 ± 10.8	0.7
C22:6 n-3, DHA	141.9 ± 18.1	2.0	132.3 ± 13.7	2.0	152.1 ± 25.2	2.1
Total SFA	2114.8 ± 149.0	30.5	2037.5 ± 256.0	30.5	2237.0 ± 102.0	30.5
Total UFA	4829.4 ± 690.0	69.5	4644.5 ± 509.0	69.5	5107.4 ± 350.0	69.5
Total MUFA	2563.3 ± 382.0	36.9	2471.5 ± 282.0	37.0	2710.6 ± 189.0	36.9
Total PUFA n-3	508.4 ± 31.4	7.3	478.6 ± 48.5	7.2	540.7 ± 57.4	7.4
Total PUFA n-6	1757.7 ± 334.0	25.3	1694.5 ± 182.0	25.4	1856.1 ± 218.0	25.3
Overall total	6944.2 ± 802.0		6682.0 ± 765.0		7344.4 ± 255.0	
n-6 : n-3 ratio	3.5	3.5	3.4			
UFA: SFA ratio	2.3	2.3	2.3			
PUFA: SFA ratio	1.1	1.1	1.1			

The results are presented as mean values ± SEM.
All values within a row are not significantly different from each other (P>0.05).

n-3 (linolenic, EPA, DPA and DHA) and n-6 (linoleic, arachidonic) classes fatty acids in this product (Table 3) were a value-added to the product as these are now regarded as nutritionally important fatty acids (Hansen, 1986; Lauritzen *et al.*, 2001; Elmore *et al.*, 2005). The presence of n-3 long chain PUFA in the product was a direct result of incorporation of a sizeable portion of marine fish (*Rastrelliger kanagurta*) as one of the raw materials. Marine fish are rich in n-3 fatty acid because their diet is made up of zooplankton and phytoplankton, a rich source of PUFA (Kyle, 2002). This could offer an added advantage, which helps to improve the content of these fatty acids in animal and animal products when the FP is being fed to livestocks. The long chain n-3 fatty acids are prized for the beneficial effects to human health and feedstuffs with added n-3 are definitely more advantageous over conventional feeds. However, the long–chain PUFA particularly the members of n-3 fatty acids are susceptible to lipid oxidation. Surprisingly, these PUFA especially EPA, DPA and DHA were detected in a substantial amount in the FP. EPA was found to be the highest among all the n-3 long chain fatty acids in the FP. Besides, the high content of n-6 PUFA detected in the FP was probably a direct result of the incorporation of more than 50% rice bran as one of the major components of raw materials. Linoleic acid (the predominant n-6 fatty acid in the product) is the major fatty acid present in the lipid fraction of the rice bran (Ito and Simpson, 1996; Rouanet *et al.*, 1993). Furthermore, rice bran contains high levels of several phytochemicals are claimed to be a good source of natural antioxidants (Krings *et al.*, 2000; Kanaya *et al.*, 2004; Iqbal *et al.*, 2005). Among these natural antioxidants are vitamin E, tocopherols, tocotrienols and the ã-oryzanol fraction, which are thought to exert anti-oxidative and protective effects on fatty acids in animal tissues (Qureshi and Peterson, 2001; Kanaya *et al.*, 2004). In addition, rice bran has been shown to improve the storage stability of foods (Iqbal *et al.*, 2005). Thus, it may have acted as a natural antioxidant during fermentation thereby improving the storage stability of FP and helping to prevent the oxidation of long-chain PUFA in FP.

CONCLUSION

The physical, chemical and microbiological properties of FP obtained in this study showed that the FP was highly consistent and reproducible. FP was rich in LAB and had a low pH. Furthermore, the presence of essential PUFA such as linoleic, linolenic, arachidonic, EPA, DPA and DHA in the FP was another important characteristic of this product. The FP was a dry-brownish mash with distinctive lime-flavoured aroma. This could have an added advantage as it would overcome the problem of unpleasant odour in feed such as fishmeal, as off-flavoured products could be a significant market disadvantage. Based on the results obtained in the present study, it indicates that FP has great potential as an animal feed. The results obtained from this study are essential for large-scale FP production for animal feed applications in the future.

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