WFL Publisher
Science and Technology

Meri-Rastilantie 3 B, FI-00980
Helsinki, Finland
e-mail: info@world-food.net

Journal of Food, Agriculture & Environment Vol.7 (3&4) : 804-810. 2009 *www.world-food.net*

Effects of brewer's by-product on the fermentation quality of ensiled total mixed ration

Md. K. Uddin [1], J. Kita [1], H. Hiraoka [2], M. Kondo [1] , S. Karita [1] and M. Goto [1]*

*[1] Faculty of Bioresources, Mie University, 1577 Kurimamachiya-cho, Tsu 514-8507, Japan. [2] Mie Science and Technology Promotion Centre, Ureshino-cho515-2322, Japan. *e-mail: goto@bio.mie-u.ac.jp, 508d1s1@m.mie-u.ac.jp*

Received 7 June 2009, accepted 28 September 2009.

Abstract

Two experiments were conducted to determine the effect of soy sauce and vinegar brewer's by-product on the fermentation characteristics of total mixed ration (TMR) silage. In Experiment 1, we examined the *in vitro* microbial growth of the following strains of lactic acid bacteria (LAB): *Lactobacillus plantarum*, *Lactococcus lactis* ssp. *lactis*, *Leuconostoc mesenteroides* ssp. *mesenteroides* and *Pediococcus acidilactici*, Clostridia, e.g. *Clostridium butyricum*, and yeasts species, e.g. *Pichia anomala*, *Pichia membranifaciens* and *Issatchenkia orientalis* in the media containing 2, 4, 6 and 8% of NaCl, because the soy sauce by-product is rich in NaCl. The growth of these strains was determined by measuring the optical density at 660 nm (OD_{660}). The growth of lactic acid bacteria strains and *C. butyricum* were significantly (p<0.01) reduced in the presence of 2% NaCl compared with the control. The growth of yeasts was also significantly (p<0.01) decreased in the presence of NaCl, except for *P. anomala* which decreased with more than 2% compared with no addition. In Experiment 2, we made 6 types of pellets using soy sauce (S) and vinegar brewer's (V) by-products and commercial concentrates (C) at different ratios of S:C (3:1 and 1:1), V:C (3:1 and 1:1) and their synergistic mixture SV:C (3:1 and 1:1). The pellets were added at 10, 20 and 30% on fresh matter basis of TMR silage and kept at room temperature for 15 days. The number of LAB was significantly (p<0.01) lower in soy sauce by-product pellet treated TMR silage compared with control, vinegar brewer's and mixture of soy sauce and vinegar brewer's by-product pellet treated TMR silages. The higher number of yeasts was found in TMR silage treated with different types of pellets compared with the control TMR silage. The addition of all by-products pellet showed low lactic acid and VFA concentrations and high pH value compared with control TMR silage. No significant difference (p> 0.05) was found in butyric acid concentration in different addition level of by-product pellet treated TMR silages not only that but also no butyric acid was found in S:C (1:1) at all addition levels and in S:C (3:1) 20% addition level. The results of this study indicate that the addition of soy sauce by-products pellet can inhibit the over fermentation as indicated by decreased lactic acid and no or low butyric acid concentration of TMR silage.

Key words: Fermentation quality, food fermentation by-products, TMR silage.

Introduction

Soy sauce and vinegar brewer's cake are among by-products produced from microbial fermentation of soybean and Japanese wine processing industries. Soy sauce cake constitutes 26.1% sugar, 10.7% NaCl with 81.5% total digestible nutrient and vinegar cake contains 53.9% protein with a low pH value of 3.88 (Mizkan Chemical Industries, Nagoya, Japan). A huge quantity of 86 and 160 metric tons (MT) of soy sauce and vinegar brewer's cake, respectively, are produced annually in Japan [12], revealing that a vast amount of waste by-product is accumulated for disposal. It is evident that organic by-products obtained from food processing plants and industries could be utilized for animal feed and hence environmental pollution and feed costs could be reduced [13]. Moreover, the use of NaCl at the early stage of ensiling has been found to inhibit the growth of undesirable microorganisms of sorghum silage [2] and alfalfa silage [19]. On the other hand, the importance of using acetic acid as a silage additive has been documented [1]. Acetic acid as an effective component minimized gas and lactic acid production and eliminated any other undesirable acid production when used with lactic acid and ethanol.

Silages may be blended with other feeds to create a total mixed ration (TMR). Farmers are encouraged to feed a TMR to stabilize microbial function and improve energy and protein utilization in the rumen [3]. More than 10 ingredients are usually formulated to create a TMR, which is stored in thick polythene bags and in an appropriate environment. Water is added to prevent the occurrence of dust and selective feeding by dairy cow. The dry matter (DM), crude protein (CP) and total digestible nutrient (TDN) contents are 50-60, 16-18 and 72-74% of DM, respectively, and silages are commercialized in transportable bag silos [17]. Since the high level of moisture at ensiling may lead to some adverse conditions, such as prolonged fermentation, excessive protein and energy loss in the silages, and ultimately to the secondary clostridial fermentation [9, 24].

Limited research has been conducted on the fermentation quality of TMR silage [6, 17, 18, 21, 23], and most of these studies are related to nutrient losses of TMR silage. Recent research has been embarked on microorganisms occurring in TMR silage that contains high amount of lactic acid during ensiling [18, 21]. Recently research has also been directed to the determination of fermentation quality with soybean card residue and wet brewer's grains as a main ingredient [23] and coffee grounds [29]. Wang and Nishino [23] reported that lactic acid production was higher in TMR silage when soybean card residue was used as one of the main ingredients. This viability of production has promoted to control the lactic acid fermentation of TMR silage at the time of ensiling. Experimental data in this regard are merger and not reliably available. Therefore, it is indispensable to investigate and find out whether the soy sauce by-product (resource of NaCl) could enhance lactic acid production during ensiling.

In this study, two laboratory experiments were conducted to examine the effect of brewer's by-product on the fermentation quality of ensiled TMR. In Exp. 1, the growth and propagation of microorganisms principally related to silage fermentation was tested to find out their inhibitory effect in NaCl concentrated medium, because soy sauce by-product contains high amount of salt and is mainly well known as a source of salt (NaCl). In Exp. 2, pellets made from brewer's by-product were added to the total mixed ration and the prepared silage was ensiled for 15 days. Thereafter, microbial counts and fermentation products were monitored.

Materials and Methods

Experiment 1- Effect of NaCl concentration on the growth of microorganisms: Typical bacteria and yeasts, generally detected in silage, were used in this study to examine their growth in the medium. The bacteria strains were *Lactobacillus plantarum* (IFO14713) [7], *Lactococcus lactis* subsp. *lactis* (IFO12007), *Leuconostoc mesenteroides* subsp. *mesenteroides* (IFO12060), *Pediococcus acidilactici* (IFO3885), *Clostridium butyricum* (IFO3858) and yeasts were *Pichia anomala*, *P. membranifacians* and *Issatchenkia orientalis* (isolated from TMR silage in this laboratory). These microbes were cultivated in the recommended media (IFO 2000) with 0, 2, 4, 6 and 8% of NaCl (w/w) at 30°C. Five replicates of bacteria and yeast cultures were incubated and grown in 10-ml test tubes and Petri plates anaerobically and aerobically, respectively. After the recommended period of incubation, the growth of the bacteria and yeasts was determined by measuring the optical density at 660 nm (OD_{660}). The optical density was adjusted with blank solution using distilled water. Additionally, *Lactobacillus plantarum* (IFO14713) was cultured in the IFO recommended (804 culture) medium containing 3, 5 and 10% fresh matter of soy sauce cake and vinegar brewer's cake. Then the culture was incubated anaerobically at 30°C for 3 days. The growing culture was then transferred in GYP-$CaCO_3$ agar medium and the quantitation of growth was enumerated in five replicates according to the plate count method [11].

Experiment 2-Effect of different types of pellet on the fermentation quality of TMR silage:

Preparation of pellet and silage: Soy sauce and vinegar brewer's cake were obtained from Mizkan Chemical Industries, Nagoya, Japan. Six types of pellet employed in this study were: 1) Soy sauce cake (S), 2) Vinegar brewer's cake (V), 3) Commercial concentrates (C) with different ratios of this three components including (i) S:C (3:1, FM/FM) and S:C (1:1), (ii) V:C (3:1) and V:C (1:1), (iii) SV:C (3:1) and SV:C (1:1) (SV = Mixture of soy sauce and vinegar brewer's cake and FM= Fresh matter).

TMR ingredients are given in Table 1. The TMR was ensiled with treatment of items of six types pellet at a rate of 10, 20 and 30% of TMR on the fresh matter basis. The silage was ensiled in 1-litre plastic bottle silo with Bunsen valves and stored in a room maintained at 22-25°C for 15 days. Treatments were made in triplicates.

Table 1. Ingredients and chemical composition of TMR used in this study.

Feed composition	Mixing ratio (%) Fresh wt.	Dry wt.
Feed ingredients		
Italian rye grass silage	11.4	6.7
Corn silage	10.4	5.4
Sudan grass silage	15.5	8.1
Alfalfa grass	3.9	5.3
Timothy hay	3.9	5.4
Brewer's grain	11.0	6.2
Dried beet pulp	8.5	12.3
Alfalfa hay cube	1.8	2.6
Mixed concentration	7.4	10.4
Ground corn	9.6	13.3
Barley	2.9	4.2
Wheat bran	3.3	4.6
Cooked bean cake	2.2	3.3
Soybean meal flake	2.9	4.2
Gluten feed	2.2	3.2
Whole cotton seed	2.2	3.3
Phosphorus mixture	0.4	0.6
$CaCO_3$	0.4	0.6
NaCl	0.1	0.3
Chemical composition		
Moisture (%)	39.60	
pH	5.19	
Lactate (%DM)	1.02	
Acetate (%DM)	0.36	
Butyrate (%DM)	ND	

TMR Total mixed ration; DM Dry matter; ND Not detected.

Microbiological and chemical analyses: The changes in the number of microorganisms were enumerated at end of ensiling. The loads of microorganisms were measured by the plate count methods [30]. According to the method, TMR silage samples (30 g) were shaken well with 270 ml sterilized distilled water, and dilutions of 10^{-1} to 10^{-8} were made with 0.85% sodium chloride solution. The colony forming units of lactic acid bacteria were determined on GYP-$CaCO_3$ agar plates [11]. The lactic acid bacteria were detected by a yellowish colony and a clear zone caused by the dissolution of $CaCO_3$. The yeasts were counted on potato dextrose agar (PDA). The plates were kept in an incubator at 30°C for 2 or 3 days. Colonies were counted as viable numbers of microorganisms (cfu/g FM).

The chemical composition of silages was analyzed from cold water extracts after 15 days of ensiled. A 30 g of silage sample was macerated in 270 ml distilled water and homogenized for 30 seconds in a blender. The resulted suspension was filtered through two layers of cheese cloth. The pH value of the filtrate was measured by using HORIBA pH meter and the filtrate was stored at -30°C until analysis of lactic acid and volatile fatty acids (VFA) concentrations. The concentrations of lactic acid and VFA of the filtrate were determined later by high performance liquid chromatography (HPLC) (Shimadzu SCR-102 (H), 12 mm I.D.×30 cm, Shimadzu Corporation, Kyoto, Japan; Goto *et al.* [5]).

***Statistical analysis*:** Data were analyzed using Microsoft Office Excelstats 2007 for Windows (Social Survey Research Information

Co. Ltd.) in a randomized block design.When significant differences occurred, means were separated by the Tukey-Kramer test [20]. The effects of types of pellet or the rate of addition level were tested by using two-way analysis of variances.Significance between the means was tested using the least significance difference (LSD).

Results

Experiment 1- Effect of NaCl concentration on growth of microbes: The OD_{660} value of the bacterial cell cultures was represented in Table 2. The growth of *L. plantarum*, *L. lactis* ssp. *lactis*, *Leu*. *mesenteroides* ssp. *mesenteroides* and *P. acidilactici* was significantly different for different concentration of NaCl. The growth of all lactic acid bacteria (LAB) species declined ($p<0.01$) due to the addition of NaCl compared with no addition with the exception of *L. plantarum* at 6 h of incubation as indicated by the OD_{660} value. The growth of *L. plantarum* significantly ($p<0.01$) decreased with the addition of NaCl in the medium and highest viability was observed at 36 h of incubation excepting for 6% NaCl addition. The highest growth of *L. lactis* was found at 36 h of incubation at 0 and 2% addition, whereas at 4, 6 and 8%, this was attained at 12 h and 48 h, respectively. Furthermore the growth of *L. lactis* was found comparatively high among all species of LAB as per OD_{660} value. The growth of *Leu. mesenteroides* ssp. *mesenteroides* and *P. acidilactici* were similar to both control and NaCl concentrated medium. The growth rate was slow compared with *L. plantarum* and *L. lactis*. Little or no growth was found at 8% NaCl addition. No differences were found between control and 2% NaCl addition in the growth of *Leu. mesenteroides* ssp. *mesenteroides* and *P. acidilactici* at 72 h of incubation.

The OD_{660} value of *C. butyricum* was significantly ($p<0.01$) low in NaCl added medium in comparison with no added medium (Table 3). The highest growth was found at 20 h of incubation in case of control medium, whereas this was attained at 24 h of incubation at 2, 4 and 6% NaCl added medium. No growth was found at 8% NaCl addition except for 16 h of incubation. Here is an interesting note that when NaCl was added in the medium even at 2% rate, the growth of *C. butyricum* decreased almost half of no added medium.

The growth of yeast species was significantly ($p<0.01$) decreased by the addition of NaCl in the medium except for *Pichia anomala* at 3 h of incubation ($p<0.05$) compared with no addition (Table 4). The OD_{660} value of *P. anomala* was decreased with addition of NaCl except for 2% addition and 24 h incubation with no addition, 2% and 4%. The highest OD_{660} value was found at 24 h incubation in both no addition and NaCl added medium. The OD_{660} value of *P. membranifaciens* significantly ($p<0.01$) decreased by the addition of NaCl in the medium in comparison with no addition except for 3 h incubation with 2% addition. The highest OD_{660} value was found at 15 h incubation in control medium and at 24 h incubation in NaCl added medium. The OD_{660} value of *Issatchenkia orientalis* analogously decreased by NaCl but a little higher value was found in comparison with other two yeast species. The highest value was found at 15 h and 24 h of incubation in case of no addition, 2% NaCl and in higher NaCl added medium, respectively.

The number of *L. plantarum* was significantly ($p<0.01$) increased in soy sauce cake and vinegar brewer's cake-treated medium compared with control medium (Table 5). The highest number was found in 5% soy sauce cake-treated medium.

Table 2. Effect of NaCl concentration in the medium on the growth (OD_{660}) of various species of lactic acid bacteria.

Incubation time (h)	NaCl concentration (% of medium) 0	2	4	6	8	SD	Significance
Lactobacillus plantarum							
6	0.01	0.00	0.00	0.00	0.00	0.00	NS
12	0.11^{a}	0.08^{b}	0.01^{c}	0.00	0.00	0.05	**
16	0.25^{a}	0.18^{b}	0.10^{c}	0.00	0.00	0.11	**
20	0.44^{a}	0.27^{b}	0.13^{c}	0.00	0.00	0.18	**
24	0.61^{a}	0.37^{b}	0.20^{c}	0.01^{d}	0.00	0.25	**
36	0.80^{a}	0.62^{b}	0.33^{c}	0.07^{d}	0.00	0.33	**
48	0.66^{a}	0.52^{b}	0.31^{c}	0.10^{d}	0.00	0.27	**
72	0.61^{a}	0.55^{b}	0.33^{c}	0.12^{d}	0.00	0.25	**
Lactococcus lactis							
6	0.81^{a}	0.69^{b}	0.25^{c}	0.00	0.00	0.35	**
12	0.82^{a}	0.66^{b}	0.60^{c}	0.05^{d}	0.00	0.34	**
16	0.78^{a}	0.66^{b}	0.56^{c}	0.12^{d}	0.00	0.31	**
20	0.81^{a}	0.66^{b}	0.55^{c}	0.15^{d}	0.00	0.31	**
36	0.93^{a}	0.72^{b}	0.54^{c}	0.40^{d}	0.00	0.32	**
48	0.76^{a}	0.62^{b}	0.51^{c}	0.48^{d}	0.02^{e}	0.25	**
72	0.78^{a}	0.62^{b}	0.50^{c}	0.44^{d}	0.01^{e}	0.26	**
Leuconostoc mesenteroides							
6	0.06^{a}	0.04^{b}	0.00	0.00	0.00	0.03	**
12	0.24^{a}	0.16^{b}	0.01^{c}	0.00	0.00	0.10	**
16	0.37^{a}	0.25^{b}	0.11^{c}	0.02^{d}	0.00	0.15	**
20	0.37^{a}	0.32^{b}	0.20^{c}	0.08^{d}	0.00	0.16	**
36	0.39^{a}	0.32^{b}	0.16^{c}	0.04^{d}	0.00	0.14	**
48	0.36^{a}	0.33^{b}	0.22^{c}	0.14^{d}	0.02^{e}	0.13	**
72	0.32^{a}	0.32^{a}	0.21^{b}	0.12^{d}	0.01^{e}	0.12	**
Pediococcus acidilactici							
6	0.06^{a}	0.03^{b}	0.00	0.00	0.00	0.23	**
12	0.15^{a}	0.10^{b}	0.01^{c}	0.00	0.00	0.06	**
16	0.19^{a}	0.16^{b}	0.02^{c}	0.00	0.00	0.09	**
20	0.23^{a}	0.19^{b}	0.05^{c}	0.00	0.00	0.10	**
36	0.35^{a}	0.31^{b}	0.14^{c}	0.01^{d}	0.00	0.16	**
48	0.37^{a}	0.33^{b}	0.22^{c}	0.05^{d}	0.01^{d}	0.15	**
72	0.41^{a}	0.44^{a}	0.28^{b}	0.07^{c}	0.00	0.18	**

OD_{660} Optical density at 660 nm. SD Standard deviation., a,b,c,d,e Different superscript means significant differences in the same row, * $p<0.05$; ** $p<0.01$. NS Non-significant.

Experiment 2- Effect of different types of pellet on the fermentation of TMR silage: The moisture content and pH of TMR silage were 39.6% and 5.19, respectively, and the concentrations of lactate and acetate were 1.02 and 0.36% as dry matter basis, respectively (Table 1). The NaCl concentrations and pH of different pellets at different addition levels were ~2.35 (%DM) and ~5.10, respectively (Table 6).

The moisture content of TMR silage was significantly ($p<0.01$) decreased by the addition of different pellets (Table 7). Among the treatments, vinegar brewer's pellet-treated TMR silage had highest moisture content followed by mixture of soy sauce and vinegar brewer's and soy sauce by-product pellets treated TMR silages. There was no effects of different pellet for decreasing the pH value of TMR silage because of the pH value was low in control TMR silage compared with pellet-

Table 3. Effect of NaCl concentration in the medium on the growth (OD_{660}) of *Clostridium butyricum*.

Incubation time	NaCl concentration (% of medium)					SD	Significance
(h)	0	2	4	6	8		
4	0.07^{a}	0.03^{b}	0.01^{c}	0.00	0.00	0.03	**
8	0.33^{a}	0.12^{b}	0.05^{c}	0.01^{c}	0.00	0.13	**
12	0.52^{a}	0.21^{b}	0.09^{c}	0.01^{d}	0.00	0.20	**
16	0.61^{a}	0.31^{b}	0.15^{c}	0.02^{d}	0.01^{d}	0.23	**
20	0.63^{a}	0.33^{b}	0.17^{c}	0.02^{d}	0.00	0.24	**
24	0.60^{a}	0.33^{b}	0.19^{c}	0.04^{d}	0.00	0.22	**

OD_{660} Optical density at 660 nm. SD Standard deviation. ^{a,b,c,d} Different superscript means significant difference in the same row. ** p<0.01.

Table 4. Effect of NaCl concentration in the medium on growth (OD_{660}) of various yeasts isolated from TMR silage.

Incubation time	NaCl concentration (% of medium)					SD	Significance
(h)	0	2	4	6	8		
Pichia anomala							
3	0.03^{a}	0.02^{ab}	0.01^{b}	0.01^{b}	0.01^{b}	0.01	*
6	0.05^{a}	0.04^{a}	0.02^{b}	0.00	0.00	0.02	**
9	0.16^{a}	0.15^{a}	0.07^{b}	0.02^{cd}	0.01^{d}	0.07	**
12	0.34^{a}	0.37^{a}	0.16^{b}	0.05^{cd}	0.02^{d}	0.15	**
15	0.63^{a}	0.63^{a}	0.36^{b}	0.12^{c}	0.03^{d}	0.26	**
24	0.86^{a}	0.86^{a}	0.81^{a}	0.46^{b}	0.17^{c}	0.28	**
Pichia membranifaciens							
3	0.04^{a}	0.03^{a}	0.02^{b}	0.02^{b}	0.00	0.01	**
6	0.16^{a}	0.13^{b}	0.08^{c}	0.04^{d}	0.01^{d}	0.06	**
9	0.46^{a}	0.37^{b}	0.24^{c}	0.11^{d}	0.03^{e}	0.16	**
12	0.78^{a}	0.66^{b}	0.49^{c}	0.22^{d}	0.07^{e}	0.27	**
15	0.98^{a}	0.90^{b}	0.75^{c}	0.45^{d}	0.14^{e}	0.32	**
24	0.94^{a}	0.90^{b}	0.88^{c}	0.71^{d}	0.43^{e}	0.19	**
Issathenkia orientalis							
3	0.11^{a}	0.07^{b}	0.02^{c}	0.01^{cd}	0.01^{d}	0.04	**
6	0.49^{a}	0.30^{b}	0.03^{c}	0.02^{cd}	0.01^{d}	0.20	**
9	0.89^{a}	0.72^{b}	0.06^{c}	0.02^{d}	0.00	0.40	**
12	1.01^{a}	0.91^{b}	0.15^{c}	0.02^{d}	0.00	0.46	**
15	1.13^{a}	1.01^{b}	0.31^{c}	0.02^{d}	0.00	0.49	**
24	1.08^{a}	0.96^{b}	0.68^{c}	0.02^{d}	0.01^{d}	0.46	**

TMR Total mixed ration; OD_{660} Optical density at 660 nm. SD Standard deviation. ^{a,b,c,d,e} Different superscript means significant difference in the same row. * p<0.05; ** p<0.01.

Table 5. Effect of soy sauce (S) and vinegar brewer's (V) by-product on the growth ($\log_{10}$cfu/g FM) of *Lactobacillus plantarum* in the medium after 3 days incubation.

Treatment	Addition level (%)			SD	Significance
	3	5	10		
Control	7.05^{c}	7.07^{c}	7.03^{c}	0.01	NS
S	7.49^{bB}	9.04^{aA}	9.02^{aA}	0.02	**
V	8.00^{aB}	8.39^{bA}	8.02^{bA}	0.01	**

cfu Colony forming unit, FM Fresh matter; SD Standard deviation. a, b, c Different superscript means significant difference in the same column. A, B Different superscript means significant difference in the same row. ** p<0.01; NS Non-significant.

treated TMR silage (Table 7). Among different treatments, the highest pH value was in soy sauce pellet-treated TMR followed by mixture of soy sauce and vinegar brewer's and vinegar brewer's pellet-treated TMR silages. In regarding addition level, no significant effect on the moisture content of different treatments of TMR silage whereas different addition level had significant (p<0.01) effect on pH changes of different pellet-treated TMR silages.

The LAB counts were low (p<0.01) in soy sauce and mixture of soy sauce and vinegar brewer's pellet but was high (p<0.01) in vinegar brewer's by-product pellet-treated TMR silages in compared with control TMR silage (Table 7). Furthermore, the LAB significantly (p<0.01) differed among different levels but not in a manner that was consistent with addition level. Yeast counts tended to be higher (p<0.01) in all pellet-treated TMR silages compared with control TMR silage (Table 7). Also yeast counts were significantly (p<0.01) different among addition levels but not in a manner that was related with addition level as like as LAB.

Regardless of levels, the lactate concentration of TMR silage was significantly (p<0.01) decreased by soy sauce, vinegar brewer's and mixture of soy sauce and vinegar brewer's by-products pellets (Table 7). The highest concentration was found in control TMR silage and the lowest concentration in S:C (3:1) pellet-treated TMR silage at 30% addition level. Among different treatments, soy sauce by-product pellet- treated silage had low lactate concentration compared with vinegar brewer's and mixture of soy sauce and vinegar brewer's by-product pellet-treated TMR silages. Similarly, the acetate concentration of different by-product pellet-treated TMR silages was significantly (p<0.01) lower compared with control TMR silage (Table 7). Among the different treatments, soy sauce by-product pellet-treated TMR silage had low acetate concentration compared to vinegar brewer's and mixture of soy sauce and vinegar brewer's by-product pellet-treated TMR silages. Furthermore, the acetate concentration was significantly (p<0.01) decreased by using high addition level except for SV:C (3:1) at 30% addition level. The low butyric acid was found in all pellet-treated TMR silages, except for S:C (1:1) pellet-treated TMR silage had no butyric acid. Among treatments, TMR silage treated with soy sauce pellet had lower (p<0.01) butyric acid concentrations than vinegar brewer's and mixture of soy sauce and vinegar brewer's pellet- treated TMR silages. There was no difference among addition levels on the effect of butyric acid concentration. The total organic acids declined significantly (p<0.01) on addition of soy sauce, vinegar brewer's and mixture of soy sauce and vinegar brewer's pellet. The highest concentration was in control TMR silage followed by vinegar brewer's, mixture of soy sauce and vinegar brewer's and lowest concentration was in soy sauce by-product pellets-treated TMR silages. The addition level was also significantly (p<0.01) decreased the total organic acids concentration. The highest concentration was found in 10% addition level followed by 20 and 30% addition level except for 10% addition level V:C (3:1) and SV:C (1:1) pellet-treated TMR silages.

Discussion

Effect of NaCl concentration on growth of microorganisms: Common salt (NaCl) has long been used for pickling and preservation of food. Shockey and Borger [19] reported that salt

Table 6. NaCl concentration and pH of TMR added with different types of pellet made from soy sauce cake (S), vinegar brewer's cake (V) and concentrates (C).

Treatment	Addition level (%)	NaCl (%DM)	pH
Control	None	0.30	5.19
S:C (3:1)[1)]	10	0.79	5.05
	20	1.57	5.08
	30	2.35	5.07
S:C (1:1)	10	0.55	5.09
	20	1.09	5.04
	30	1.60	5.10
V:C (3:1)	10	not detected	5.00
	20	not detected	4.84
	30	not detected	4.79
V:C (1:1)	10	not detected	4.99
	20	not detected	4.94
	30	not detected	4.85
SV:C (3:1)	10	0.42	4.93
	20	0.84	5.04
	30	1.26	4.90
SV:C (1:1)	10	0.29	5.03
	20	0.58	5.04
	30	0.85	4.97

TMR Total mixed ration; DM Dry matter. [1)] A pellet of S:C (3:1) means a mixture of soy sauce cake and concentrates by 75% and 25% of fresh matter basis, respectively.

effectively inhibits the growth of epiphytic lactic acid bacteria and butyric acid bacteria. Moreover sodium and chlorine ions are essential to the maintenance of osmolarity in microorganisms and plants. Increasing the concentration of these components could also affect the growth of non-salt-organisms to maintain their cellular processes [19]. In this study, almost all genera of lactic acid bacteria were used that are closely related with silage fermentation and the growth of *Lactobacillus plantarum* was significantly ($p<0.01$) decreased with 2% addition of NaCl in the medium and the growth was seen to gradually fall with increasing concentration. Analogously the growth of *Lactococcus lactis* was found significantly ($p<0.01$) decreased using NaCl in the medium. The growth of *Leuconostoc mesenteroides* and *Pediococcus acidilactici* were also observed significantly ($p<0.01$) decreased with increasing NaCl concentration in the medium. However, the growth pattern was delayed and was associated very similar to *L. plantarum* at the beginning. The enhanced growth of *L. plantarum*, *L. lactis* ssp. *lactis*, *P. acidilactici* and *Leu. mesenteroides* in the presence of NaCl was attributable to control lactic acid fermentation. *Leu. mesenteroides* was very much sensitive to NaCl and this is in good agreement with the findings of Korkeala *et al.* [8] but contradicts with the results reported by Shockey and Borger [19]. They observed that the growth of LAB was tolerant up to 6.5% NaCl in the medium.

The growth of *C. butyricum* was significantly ($p<0.01$) declined by using of NaCl in the medium. The growth was found to have reduced to almost half when 2% NaCl was used in the medium and at 8% concentration there was stationary growth of *C. butyricum*. The reduction in growth of butyric acid bacteria due to NaCl was similar to the opinion of Shockey and Borger [19]. Evidence has also indicated that NaCl inhibited the growth of butyric acid bacteria in sorghum silage [2].

The growth of *P. anomala* was not enhanced by 2% NaCl in the medium whereas the growth of *P. membranifaciens* and *I. orientalis* was significantly ($p<0.01$) decreased with the same concentrated level. This means that the later two yeast species were more sensitive to NaCl compared to previous species. Although the tolerance of yeasts to NaCl concentration and lactate is well documented [10] and researches so far have been done on the effect of NaCl on yeast growth by Weinberg [25] and Cai *et al.* [2] gives the impression that more studies are imperative. In the present study the growth of yeast was found to have significantly fallen when 2% NaCl was added in the medium except for *P. anomala*.

Effect of different types of pellet type on the fermentation quality of TMR silage: Additive treatment was beneficial for inhibiting the growth of lactic acid bacteria in TMR silages ensiled for 15 days. However, there was no effect of vinegar brewer's by-product pellet on inhibiting the growth of lactic acid bacteria in TMR silage ensiled for same days. This result suggests that NaCl (soy sauce

Table 7. Microbial counts and chemical composition of TMR silage treated with different types of pellet made from soy sauce cake (S), vinegar brewer's cake (V) and concentrates (C) after ensiling for 15 days.

Treatment	Addition level	Moisture	pH	LAB	Yeasts	Lactate	Acetate	Butyrate	Total
	%	%		$\log_{10}$cfu/g FM		% of DM			
Control	None	36.4	4.71	6.04	4.03	2.31	0.96	0.01	3.28
S:C (3:1)[1)]	10	29.0	5.00	6.31	4.37	1.26	0.50	0.03	1.79
	20	31.9	5.09	4.73	4.54	1.05	0.39	0.00	1.47
	30	29.6	5.09	4.77	4.21	0.89	0.33	0.01	1.29
S:C (1:1)	10	33.3	4.98	5.34	4.28	1.39	0.54	0.00	1.93
	20	29.5	5.07	5.11	4.36	1.13	0.41	0.00	1.58
	30	27.0	5.10	4.93	4.40	0.93	0.32	0.00	1.29
V:C (3:1)	10	35.2	4.80	6.26	4.19	1.01	0.50	0.01	1.52
	20	35.0	4.79	6.75	4.40	1.47	0.83	0.04	2.33
	30	33.9	4.78	6.67	4.50	1.20	0.79	0.02	2.02
V:C (1:1)	10	35.7	4.85	6.72	4.20	1.83	0.84	0.05	2.71
	20	33.8	4.88	6.12	4.31	1.41	0.71	0.03	2.15
	30	31.8	4.91	5.75	4.27	1.02	0.60	0.00	1.62
SV:C (3:1)	10	35.3	4.92	6.13	4.62	1.63	0.72	0.05	2.41
	20	34.4	4.98	5.89	4.44	1.21	0.58	0.00	1.78
	30	32.0	5.04	5.45	4.51	0.91	0.49	0.02	1.44
SV:C (1:1)	10	35.1	4.86	6.40	4.07	1.66	0.75	0.01	2.42
	20	33.3	5.04	5.80	4.47	1.61	0.80	0.04	2.44
	30	32.2	5.04	5.77	4.30	1.10	0.50	0.02	1.62
SD		0.007	0.03	0.07	0.07	0.21	0.05	0.02	0.19
Significance	T	**	**	**	**	**	**	**	**
	A	NS	**	**	**	*	**	NS	**
	T×A	**	**	**	**	**	**	**	**

TMR Total mixed ration; LAB Lactic acid bacteria; cfu Colony forming unit; FM Fresh matter; DM Dry matter. [1)] A pellet of S:C (3:1) means a mixture of soy sauce cake and concentrates by 75 and 25% of fresh matter basis, respectively. S Standard deviation; T Treatments; A Addition level. * $p<0.05$; ** $p<0.01$; NS Non-significant.

by-product) may be a greater determinant in inhibiting the growth of epiphytic lactic acid bacteria at the early stage of ensiling. In Experiment 1, the growth of LAB decreased when 2% NaCl (FM basis) was used in the medium. This result is in agreement with several workers [4, 19, 26, 28]. Woolford [28] reported that the microbial growth was inhibited with a reduced pH and acid production when alfalfa silage was treated with NaCl. Furthermore, Shockey and Borger [19] found that salt effectively inhibited the growth of butyric acid bacteria and, apparently, epiphytic LAB. Whereas, high LAB and reduced pH were observed at an early stage of ensiling when the sorghum silage was treated with 4% NaCl [2]. The presence of salt-tolerant or halophilic LAB in forage and the addition of NaCl enhanced the lactic acid fermentation and inhibited the growth of butyric acid bacteria during ensiling.

The number of yeasts was high ($p<0.01$) in all pellet-treated TMR silages compared with control TMR silage. The result is not clear but maybe the low number of LAB in soy sauce by-product pellet-treated TMR silages, although no relation was found with vinegar brewer's by-product pellet treatment. Because LAB fail to produce organic acids as well as lactic acid from sugar, the pH of silage will increase and the residual sugars are used by undesirable microorganisms as well as silage yeasts [14]. Furthermore, yeasts can grow in silages of pH 3.5 and for most silage species the optimum is between pH 3.5 and 6.5 [27].

The effect of vinegar brewer's by-product pellet was negative on controlling the yeast population in TMR silage because high number of yeasts was found in vinegar brewer's by-product-treated TMR silage compared with control TMR silage. In previous studies, the addition of acetic acid had variable effects on inhibiting the growth of yeast species. For example, Moon [15] reported that high (<0.6%) concentration of acetic acids had inhibitory effects on yeast growth in the medium.

Lactic acid and butyric acid were the principal fermentation products of TMR silage in this study. Because the activity of aerobic microorganisms were increased and silage would be deteriorated when the lactic acid concentration was high in TMR silage at end of ensiling [14]. Furthermore, production of butyric acid means the clostridial activity occurred and the silage would be undergone to secondary fermentation. However, in our study, low lactic acid concentrations were found in all pellet-treated TMR silages as compared with control TMR silage. Reports are not available on low lactic acid concentration in silage fermentation and this is the first report on the effect of by-products as additives on TMR silage fermentation. The effect of soy sauce by-product pellets on inhibiting the lactic acid production was high compared with vinegar brewer's pellets and mixture of soy sauce and vinegar brewer's pellet-treated TMR silages. The result is unknown but may be effect of NaCl content of soy sauce by-product pellet and this is in good agreement with Shockey and Borger[19] and Woolford[28]. Furthermore, the low lactic acid concentration of silages concurs with the findings of Umana *et al.* [22] and is typical of tropical grass silages that have undergone heterolactic fermentation. Cai *et al.* [2] reported that the lactic acid production was high in NaCl-treated silage compared with control silage and the silage was well preserved. The butyric acid was not produced in S:C (1:1) ratio's pellet-treated TMR silage. This may be the effect of NaCl (soy sauce by-product pellet) although some butyric acid was produced in S:C (3:1) at 30% addition level. This result is supported by Woolford [28], Shockey and Borger [19] and Cai *et al.* [2] who reported a consistent effect of NaCl on inhibiting the activity of butyric acid bacteria in sorghum silage fermentation. No effect of vinegar brewer's by-product and mixture of soy sauce and vinegar brewer's by-product pellets was found on controlling the butyric acid fermentation of TMR silage.

Conclusions

All levels of NaCl used in this study effectively inhibited the growth of LAB and *Clostridium butyricum* in medium. The growth of yeast species was also effectively decreased using (2, 4, 6 and 8%) NaCl in medium, but high amount was most effective and yeast species were more tolerant than *C. butyricum* and LAB. Addition of soy sauce by-product pellets in TMR silage effectively inhibited the number of LAB but not yeasts, whereas higher number of LAB and yeasts was found in addition of vinegar brewer's by-products to TMR silage. Lower lactic acid and butyric acid concentration was found in soy sauce by-products pellet-treated TMR silages compared to control. In addition, increasing the level of soy sauce by-product pellets decreased lactic acid concentration and no or low butyric concentration in TMR silage. From the above results, it is recommended that 20% fresh matter of soy sauce by-product pellet was more effective to enhance the fermentation in context of low lactic acid and no butyric acid concentration in TMR silage.

References

[1]Byers, F.M., Goodrich, R.D. and Meiske, J.C. 1982. Influence of acetic acid, lactic acid and ethanol on the fermentation of corn silage. J. Anim. Sci. **54**:640-648.

[2]Cai, Y., Ohmomo, S., Ogawa, M. and Kumai, S. 1997. Effect of NaCl-tolerant lactic acid bacteria and NaCl on the fermentation characteristics and aerobic stability of silage. J. Appl. Microbiol. **83**:307-313.

[3]Coppock, C.E., Bath, D.L. and Jr. Harris, B. 1981. From feeding to feeding system. J. Dairy Sci. **64**:1230-1249.

[4]Georing, H.K. and Gordon, C.H. 1973. Chemical aids to preservation high moisture feed. J. Dairy Sci. **56**:1347-1351.

[5]Goto, M., Yokoe, Y., Takabe, K., Nishikawa, S. and Morita, O. 1993. Effects of gaseous ammonia on chemical and structural features of cell walls in spring barley straw. Anim. Feed Sci. Technol. **40**:207-221.

[6]Hiraoka, K., Yamamoto, S., Koide Y., Tanaka, Y., Inui, S. and Urakawa, S. 2005. Controlling effect of fermented rice plant on rapped TMR and its aerobic deterioration. J. Jpn. Soc. Grassl. Sci. **51**:124-125.

[7]Institution for Fermentation Osaka 2000. List of Cultures. 11th edn. Appendix 2. List of Media. The Business Center of Academic Societies Japan, Tokyo, pp. 587-607.

[8]Korkeala, H., Alanko, T. and Tiusanen, T. 1992. Effect of sodium nitrate and sodium chloride on growth of lactic acid bacteria. Acta Vet. Scand. **33**(1):27-32.

[9]Mahanna, W.C. 1993. Troubleshooting silage problems. 4-State Applied Nutrition Conference, June 29-30, La Crosse, WI.

[10]Masui, M., Onishi, H. and Unemoto, T. 1979. Halophilic Microorganisms. Yishiyakusyupan, Tokyo, pp. 313-348.

[11]Masuko, T., Okada, S., Uchimura, T. and Awaya, K. 1992. Effects of inoculation with lactic acid bacterial culture at ensiling on the fermentative quality and flora of lactic acid bacteria of grass silage. J. Grassl. Sci. **63**:1182-1187.

[12]Ministry of Agriculture, Forestry and Fisheries 1993. The 69th Statistical Yearbook of Ministry of Agriculture, Forestry and Fisheries. (In Japanese).

[13]Ministry of Agriculture, Forestry and Fisheries 1994. Handbook of Circulating Feed. (In Japanese).

[14]McDonald, P., Henderson, N. and Heron, S. 1991. The Biochemistry of Silage. Chalcombe Publications, Great Britain, pp. 83-151.

[15]Moon, N.J. 1983. Inhibition of the growth of acid tolerant yeasts by acetate, lactate, propionate and their synergistic mixtures. J. Appl. Bacteriol. **55**:453-460.
[16]Nishino, N. 2006. Ensiled total mixed ration. A non-conventional silage supporting animal production in Japan. Proc. 2nd China-Japan-Korea Sym. Grassl. Agric. Anim. Prod., Chinese Soc. Grassl. Sci., Lanzhou, China, pp. 193-197.
[17]Nishino, N., Harada, H. and Sakaguchi, E. 2003. Evaluation of fermentation and aerobic stability of wet brewers' grains ensiled alone or in combination with various feeds as a total mixed ration. J. Sci. Food Agric. **83**:557-563.
[18]Nishino, N., Wada, H., Yoshida, M. and Shiota, H. 2004. Microbial counts, fermentation products, and aerobic stability of whole crop corn and a total mixed ration ensiled with and without inoculation of *Lactobacillus casei* or *Lactobacillus buchneri*. J. Dairy Sci. **87**:2563-2570.
[19]Shockey, W.L. and Borger, D.C. 1991. Effect of salt on fermentation of alfalfa. 2. Treatment with sodium chloride, *Clostridium butyricum*, and lactic acid bacteria. J. Dairy Sci. **74**:160-166.
[20]Steel, R.G.D. and Torrie, J.H. 1980. Principles and Procedures of Statistical Analysis. MacGraw Hill, New York.
[21]Taylor, C.C., Ranjit, N.J., Mills, J.A., Neylon, J.M. and Kung, L.Jr. 2002. The effect of treating whole-plant barley with *Lactobacillus buchneri* 40788 on silage fermentation, aerobic stability, and nutritive value for dairy cows. J. Dairy Sci. **85**:1793-1800.
[22]Umana, R., Staples, C.R., Bates, D.B., Wilcox, C.J. and Mahanna, W.C. 1991. Effects of a microbial inoculants and (or) sugarcane molasses on the fermentation, aerobic stability and digestibility of bermudagrass ensiled at two moisture contents. J. Anim. Sci. **69**:4588-4601.
[23]Wang, F. and Nishino, N. 2008. Resistance to aerobic deterioration of total mixed ration silage: Effect of ration formulation, air infiltration and storage period on fermentation characteristics and aerobic stability. J. Sci. Food Agric. **88**:133-140.
[24]Ward, R. 1996. Interpretation of Silage Quality Values. Cumberland Valley Analytical Survices.
[25]Weinberg, Z.G., Ashbell, G., Hen, Y. and Azricli, A. 1993. The effect of applying lactic acid bacteria at ensiling on the aerobic stabling of silages. J. Appl. Bacteriol. **75**:512-518.
[26]Wilson, J.K. 1948. Changes occurring in silage as effected by salt. J. Am. Soc. Agron. **40**:901.
[27]Woolford, M.K. 1976. A preliminary investigation into the role of yeasts in the ensiling process. J. Appl. Bacteriol. **41**:29-36.
[28]Woolford, M.K. 1978. Antimicrobial effects of mineral acids, organic acids, salts and sterilizing agents in relation to their potential as silage additives. J. Br. Grassl. Soc. **33**:131-136.
[29]Xu, C.C., Cai, Y., Zhang, J. G. and Ogawa, M. 2007. Fermentation quality and nutritive value of a total mixed ration silage containing coffee grounds at ten or twenty percent of dry matter. J. Anim. Sci. **85**:1024-1029.
[30]Yamamoto, K.S., Utagawa, S., Kodama, T. and Morichi, T. 1986. Isolation Methods for Microorganisms. R and D Planning,Tokyo, pp. 435-444. (in Japanese).