Technical paper

Impact of *Lactobacillus paracasei* HD1.7 as a Starter Culture on Characteristics of Fermented Chinese Cabbage (*Brassica rapa* var. *pekinensis*)

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Lactobacillus paracasei HD1.7, an isolate from Chinese fermented cabbage and producer of bacteriocin, was used as a starter culture to estimate the impact on characteristics of fermented cabbage. The results of quantitative real-time PCR showed that in *Lb.paracasei* HD1.7 fermented sample, the total bacteria number was significantly higher during the initial and medium stages. *Lb.paracasei* HD1.7 significantly increased the final concentration of lactate (7.13 g/L), mannitol (19.41 g/L) and seven amino acids while kept vitamin C more stable (43.20 mg/kg). Meanwhile, nitrite was lower than 0.16 mg/kg throughout the whole fermentation process. The fermented cabbage with *Lb.paracasei* HD1.7 was more favorable by consumers in terms of crisp, sourness, aroma, bitterness, stink, stale flavor and overall acceptability. *Lb.paracasei* HD1.7 fermented cabbage had more nutritive values and health-promoting effects. This suggested *Lb.paracasei* HD1.7 a practical potential in industrially production as a starter culture of fermented cabbage and other fresh vegetables.

Keywords: *Brassica rapa* var. *pekinensis*, cabbage fermentation, starter culture, *Lactobacillus paracasei*, quantitative real-time PCR, fermentation characteristics

Introduction

Chinese cabbage (*Brassica rapa* var. *pekinensis*, hereinafter referred to as cabbage) is a kind of very common vegetable, which is widely planted in Asia and Europe. In autumn and winter, fresh but surplus cabbage is usually made into fermented cabbage, i.e. suan cai (sour vegetable in Chinese) which is nutritious and popular. Fermented cabbage is a very traditional and local food in Asia, especially in northeast China. The production process of fermented cabbage is similar to kimchi in Korea or sauerkraut in Germany, which relies on the fermentation by LAB (lactic acid bacteria). During the traditional producing process, cabbage is soaked into salt water at ambient temperature for weeks. Naturally occurring LAB transferred reducing sugar to organic acids, amino

acids and other flavor substances (Wu *et al.* 2012). The sensory quality of fermented vegetable is unstable due to the variety in species and amount of epiphytic LAB. Meanwhile, nitrite produced by nitrate reducing bacteria during vegetable fermentation potentially threat consumers' health (Paik and Lee 2014).

The use of LAB as starter cultures has been considered to ferment fresh vegetables into quality uniform and healthy food in decade. Several LAB such as *Leuconostoc mesenteroides*, *Leu. citreum*, and *Lactobacillus plantarum* have been applied in kimchi fermentation for quality development (Leal-Sánchez *et al.* 2003; Cho *et al.* 2014; Jo *et al.* 2014). LAB strain *Lb.delbrueckii* and *Lb.paracasei* have been used as mixed starter cultures to improve the characteristics of fermented cabbage (Han, Yi *et al.* 2014). The

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LAB inoculated into the vegetable fermentation produces more nutritive metabolites including lactate, acetate, amino acids, mannitol, and so on (Jung *et al.* 2012; Jo *et al.* 2014). Moreover, starter LAB generates more acidic conditions which results in inhibition of pathogenic bacteria (Chang and Chang 2011) and decomposition of nitrite (Han *et al.* 2014; Paik and Lee 2014).

Though the bacterial succession and metabolites production during kimchi fermentation with LAB starter is relatively well studied by means of high-throughput sequencing and metabolomics techniques (Jung *et al.* 2012; Jeong *et al.* 2013), the impact of LAB starter on characteristics of fermented cabbage is barely known. In this research, *Lb.paracasei* HD1.7 was applied in cabbage fermentation as a starter culture. This strain was isolated from the liquid sample during cabbage fermentation which permitted its good compatibility as a starter (Lei *et al.* 2007). Several studies have been conducted on strain HD1.7, such as the purification and characteristics of bacteriocin (Ge *et al.* 2009), the optimization of bacteriocin producing medium (Ge *et al.* 2011a), the establishment of genetic transformation system (Ge *et al.* 2011b) and the mechanism of quorum sensing (Ge *et al.* 2011c). Comparable analyses was conducted between *Lb.paracasei* HD1.7 starter and non-starter, i.e. spontaneously fermented cabbage, including changes in total bacteria number, pH profile, lactate, acetate, mannitol, vitamin C, nitrite and amino acids as well as sensory estimation. This research aimed to get a better understanding about how LAB starter influenced the characteristics of fermented cabbage and subsequently explore the potential application in producing commercial fermented cabbage or other fresh vegetable with more nutritive values and pleasant flavors.

Materials and Methods

Microorganism *Lb.paracasei* HD1.7 used as a starter culture was isolated from the fermentation liquid of commercial product of fermented cabbage (Fengyuan Food Company Limited, Qiqihaer, China) (Lei *et al.* 2007).

Medium Liquid MRS used for cultivation of *Lb.paracasei* HD1.7 consisted (g/L): peptone 10, beef extract 10, yeast extract 5, K_2HPO_4 2, ammonium citrate 2, sodium acetate 5, glucose 20, $MgSO_4 \cdot 7H_2O$ 0.58, $MnSO_4 \cdot 4H_2O$ 0.25, tween 80 1 mL and pH 6.2–6.4.

Starter culture preparation *Lb.paracasei* HD1.7 was cultured in MRS liquid medium at 30°C for 24 h. The bacterial cells were harvested by centrifugation and washed three times with 0.9% sodium chloride solution and then adjusted to a concentration of 10^8 cells/mL for starter inoculation.

Preparation of fermented cabbage Several cabbages with a total weight of 10 kg were washed three times with tap water, drained and then laid into a 30 L tank layer by layer as tight as possible. The cabbages were soaked in 17.5 L 3% sodium chloride solution. The starter culture of *Lb.paracasei* HD1.7 cells was

inoculated into the tank with a final concentration of 1.0×10^7 cells/g cabbage, i.e. approximate 5.7×10^6 cells/mL. The same treatment without inoculation of starter culture, i.e. spontaneous cabbage fermentation was set as a control. The fermentation period was 25 days at 17–20°C. Samples of fermented cabbage were taken at one day interval for characteristics evaluation.

Quantitative determination of 16S rRNA gene copies Quantitative real-time PCR (Applied Biosystems 7500, Life Tech, USA) was performed in triplicate to estimate the 16S rRNA gene copy number of total bacteria during cabbage fermentation. The total genomic DNA from pellets of 1 mL fermentation liquid was extracted in triplicate using a DNA Kit (KG203-02, TIANGEN, China). A standard curve was obtained according to the number of a bacterial strain carrying the 16S rRNA gene of *Lb.plantarum* HDRS1 (GenBank accession number DQ141558). The 16S rRNA gene was amplified and copy numbers were calculated based on the method of (Jung *et al.* 2011). Although the copy numbers determined by this method were not exactly equal to the cell numbers, we employed the PCR method because of a convenient one to monitor cell growth in a fermentation tank.

Characteristics measurement of fermented cabbage The pH values were directly measured using pH meter (Delta 320A, Mettler Toledo, Switzerland). L-8800 analyzer (Hitaichi, Japan) was used for determining the concentration of amino acids. The concentration data of lactate and acetate was obtained from HPLC (LC20A, Shimadzu, Japan). The pyruvate, 1,6-diphosphate fructose and mannitol was measured based on previously reported methods, respectively (Nigam 1962; Du and Wu 1993; Bok and Demain 1977). The activity of pyruvate kinase and phosphofructokinase was determined using PK assay kit and PFK assay kit (NY2 and NY3, Suzhou Comin Biotechnology Co., Ltd., China). Vitamin C was assayed based on Zhu's method (Zhu *et al.* 2002). Nitrite was measured using nitrite assay kit (A038, Nanjing Jiancheng Bioengineering Institute, China).

Sensory evaluation of fermented cabbage Samples of fermented cabbages were supplied to 15 ordinary people (6 male, 9 female, 20–50 years old). Seven sensory parameters, i.e. crisp, sourness, aroma, bitterness, stink, stale flavor and overall acceptability were evaluated ranging from 1 to 5 point which stands for negligible, little, medium, strong and extraordinary strong.

Statistical analysis We have conducted the independent experiments three times. The results in this manuscript were derived from one of these three independent experiments. The data were presented as the mean and standard error of three replicates of the independent experiment. Differences between groups were compared using ANOVA and Tukey's test. Differences were considered significant when p value was less than 0.05. Statistical data were analyzed by JMP software (SAS Institute Inc., Version 9.0.2).

Results

The pH profiles and numbers of 16S rRNA gene copies during cabbage fermentation The general pH profiles of cabbage samples fermented by *Lb.paracasei* HD1.7 over 25 days were similar to those of the control, i.e. spontaneous cabbage fermentation (Fig. 1). The pH values decreased rapidly from 5.2 – 5.3 to around 3.5 within the first seven days of the fermentation process and then remained stable until fermentation was complete (3.3 – 3.4). However, along with the inoculation of *Lb.paracasei* HD1.7 as a starter culture, the pH values decreased more dramatically and the final value was slightly lower (3.27).

The changes in 16S rRNA gene copy numbers of bacteria was calculated according to a standard curve (*Lb.paracasei* HD1.7 as a starter culture, $R^2 = 0.993$; control, $R^2 = 0.991$) derived from the 16S rRNA gene cloning of *Lb.plantarum* HDRS1. Generally speaking, the bacterial abundance increased while the pH values inversely decreased in all samples (Fig. 1). As the added strain HD1.7 thrived in the initial and middle stage of the fermentation process, the 16S rRNA gene copies in *Lb.paracasei* HD1.7 fermented samples were significantly higher than the non-starter control. The similar results have been obtained in fermented kimchi with *Leu.mesenteroides* as starter cultures (Jung *et al.* 2012). Nevertheless, as the fermentation progressed, take the 19th and 25th day for example, the copy numbers in control samples (9.8×10^8 , 1.1×10^9) surpassed those in *Lb.paracasei* HD1.7 fermented samples (7.7×10^8 , 7.8×10^8), respectively.

Content changes in metabolites related to lactate-production during cabbage fermentation In order to investigate the impact of the starter *Lb.paracasei* HD1.7 on characteristics of fermented cabbage, content changes in metabolites were detected and analyzed especially those related to lactate production. As well known, lactate is the major product in both heterofermentation and homofermentation by LAB. As expected, all along the fermentation process the lactate content was significantly higher in *Lb.paracasei* HD1.7 fermented samples (terminal concentration 7.13 g/L) compared to that in the control (terminal concentration 5.27 g/L) (Fig. 2). This resulted in more acidic conditions which coincided with pH profiles (Fig. 1). *Lb.paracasei* HD1.7 belongs to homofermentative lactic acid bacterium which is not capable of producing acetate and ethanol. Therefore, acetate and ethanol could be detectable but lower in *Lb.paracasei* HD1.7 fermented samples than those in spontaneously fermented cabbage samples (Fig. 2 and Fig. 3).

Content changes in vitamin C, mannitol and nitrite during cabbage fermentation As was shown in Fig. 4, vitamin C content was consistently higher (43.2 mg/kg) than that in spontaneously fermented samples (38.3 mg/kg). Mannitol concentration increased as fermentation progressed inversely related to the decrease in pH values (Fig. 1 and Fig. 4). The final concentration of mannitol in *Lb.paracasei* HD1.7 fermented and spontaneously fermented samples were 19.4 g/L and 7.4 g/L, respectively.

Fig. 1. Changes in pH and 16S rRNA gene copy as the total bacteria number during cabbage fermentation.

Fig. 2. Changes in lactate and acetate during cabbage fermentation.

Fig. 3. Changes in ethanol during cabbage fermentation.

The nitrite concentration in control samples were extremely high during the first 5 days and reached peak value on the third day (33.4 mg/kg) (Fig. 5A) then steadily decreased to 0.18 mg/kg (Fig. 5B). Contrastingly, throughout the whole fermentation the nitrite concentration in *Lb.paracasei* HD1.7 fermented samples were under 0.16 mg/kg with barely any fluctuation (Fig. 5).

Fig. 4. Changes in vitamin C and mannitol during cabbage fermentation.

Fig. 5. Changes in nitrite during cabbage fermentation.

Terminal content of amino acids in fermented cabbage
Seventeen amino acids were detected and their terminal contents were compared between *Lb.paracasei* HD1.7 fermented and spontaneously fermented cabbage (Table 1). The terminal concentration of seven out of seventeen amino acids, including threonine, serine, glutamic acid, tyrosine, phenylalanine, histidine and lysine, as well as total amino acids was significantly higher in *Lb.paracasei* HD1.7 fermented cabbage. There were no significant differences in concentration of other nine amino acids between *Lb.paracasei* HD1.7 fermented and spontaneously fermented cabbage. Cystine was an exception which was statistically lesser in HD1.7 fermented sample.

Sensory of fermented cabbage Besides nutritive value, sensory parameter is mostly important to evaluate fermented food. Seven sensory parameters were estimated by fifteen ordinary people in this research (Fig. 6). Except for crisp ($P = 0.2868$), *Lb.paracasei* HD1.7 fermented samples were significantly better than control in terms of six parameters, including sourness ($P = 0.0005$), aroma ($P = 0.0077$), bitterness ($P = 0.0281$), stink ($P = 0.0002$), stale flavor ($P < 0.0001$) and overall acceptability ($P = 0.0096$). This result suggested strain HD1.7 make the fermented cabbage more popular by consumers than spontaneously fermented ones.

Table 1. Terminal content and variance analysis of free amino acids in fermented cabbage.

Amino acid	Concentration $\pm$ SE $\times 10^{-2}$ (mg/100 g)		P
	HD1.7	Control	
Aspartic acid	1.43 $\pm$ 4.71	1.36 $\pm$ 5.77	0.2302
Threonine	18.70 $\pm$ 8.16	18.00 $\pm$ 10	0.0010
Serine	3.63 $\pm$ 4.71	2.56 $\pm$ 5.77	<0.0001
Glutamic acid	7.93 $\pm$ 4.71	2.26 $\pm$ 5.77	<0.0001
Glycine	3.16 $\pm$ 4.71	3.16 $\pm$ 5.77	1
Alanine	13.66 $\pm$ 4.71	13.76 $\pm$ 5.77	0.1012
Cystine	0.94 $\pm$ 4.71	0.99 $\pm$ 5.77	0.0003
Valine	6.83 $\pm$ 4.71	6.76 $\pm$ 5.77	0.2302
Methionine	1.36 $\pm$ 4.71	1.36 $\pm$ 5.77	1
Isoleucine	3.83 $\pm$ 4.71	3.76 $\pm$ 5.77	0.2302
Leucine	6.20 $\pm$ 0	6.13 $\pm$ 5.77	0.1012
Tyrosine	0.81 $\pm$ 4.71	0.79 $\pm$ 5.77	0.0048
Phenylalanine	3.83 $\pm$ 4.71	3.56 $\pm$ 5.77	0.0048
Histidine	0.66 $\pm$ 4.71	0.00000	<0.0001
Arginine	0.0000	0.00000	1
Proline	16.43 $\pm$ 4.71	16.36 $\pm$ 5.77	0.2302
Lysine	5.50 $\pm$ 8.16	5.03 $\pm$ 5.77	0.0022
Total free amino acids	94.94 $\pm$ 21.36	85.91 $\pm$ 41.49	<0.0001

Fig. 6. Sensory evaluation of fermented cabbage (n = 6).

Discussions

In fermented food, pH values directly reveal the acidity. In this research, the addition of starter culture *Lb.paracasei* HD1.7 led to a more considerable decrease and a lower final pH value. Similar conclusion have been reported by Han *et al.* that the addition of *Lactobacillus* strains as starter cultures resulted in lower acidity in fermented cabbage (Han *et al.* 2014). A quantitative real-time PCR was applied based on the 16S rRNA gene copies to estimate the total bacteria number during cabbage fermentation. In *Lb.paracasei* HD1.7 fermented samples, the reason why the 16S rRNA copy numbers decreased after increase might be that more sever acidity inhibited the growth of many Gram-negative bacteria as Han *et al.* reported previously (Han *et al.* 2014). The other probable reason

was *Lb.paracasei* HD1.7 could produce bacteriocins which prevented spoilage and over-ripening of fermented cabbage by inhibiting the growth of food-borne pathogens (Ge *et al.* 2009). Meanwhile, the nitrite content was significantly lower in HD1.7 fermented samples than that in the control (Fig. 5) which suggested the possibility of the inhibitory activity of HD1.7 against nitrate reducing bacteria. It could be concluded that the addition of *Lb.paracasei* HD1.7 significantly influenced the pH profiles and total bacterial numbers, which did not coincided with that in kimchi fermentation when *Leu.mesenteroides* was used as a starter. Jung *et al.* reported that the final pH values and 16S rRNA gene copies reached almost similar values after 18 days fermentation in regardless of starter inoculation (Jung *et al.* 2012).

This paper comprehensively studied the combining changes in metabolites and cell numbers of bacteria together to investigate the effect of LAB as a starter culture on characteristics of fermented cabbage. The metabolites such as carbohydrates, organic acids and amino acids produced by microorganisms during fermentation not only principally determine the flavors and tastes but are also main nutritious components of fermented vegetable food (Jeong *et al.* 2013; Cho *et al.* 2014). Except for the changes in lactate and acetate described in the Results section, we also investigated the dynamic changes in pyruvate kinase and 1,6-diphosphate fructose kinase together with their catalysis products pyruvate and 1,6-diphosphate fructose, which existed in the critical step of lactate homofermentation. Either the enzyme activities or product concentrations were slightly higher in *Lb.paracasei* HD1.7 fermented samples than those in the control (data not shown). It was thought that throughout the cabbage fermentation process lactate homofermentation was consistently more predominant resulted from the inoculation of starter culture *Lb.paracasei* HD1.7. This conclusion did not coincide with the investigations derived from kimchi fermentation. Chang and Chang as well as Jung *et al.* reported that during the early and middle kimchi fermentation stages heterofermentative LAB predominates under weaker acidic condition (Jung *et al.* 2012; Chang and Chang, 2011). As the fermentation continued, homofermentative LAB prevented the predominance and became the majority species. Further studies on bacterial succession and metabolomics should be conducted to figure out the dynamic mechanism during fermented cabbage ripeness.

Fresh vegetables are main source of vitamins especially vitamin C which is essential to body health. During the ripening process of fermented food vitamin C is easily to be damaged by oxidization. It was reported that vitamin C was more stable under more acidic condition in pickle (Su and Chen 2001). In *Lb.paracasei* HD1.7 fermented samples lower pH values meant more acidic conditions, vitamin C content was consistently higher than that in spontaneously fermented samples (Fig. 4). Mannitol, a naturally occurring six carbon polyol that contributes to a

refreshing taste with non-carcinogenic properties, can be produced by LAB throughout fructose reduction (Wisselink *et al.* 2002). Apparently, fermented cabbage with starter culture *Lb.paracasei* HD1.7 had more mannitol compared to the control, suggesting that the application of strain HD1.7 as starter was helpful to produce fermented cabbage with more nutritive value. This mannitol-producing function of *Lb.paracasei* HD1.7 was similar to other LAB in kimchi fermentation (Yun *et al.* 1996; Jeong *et al.* 2013).

Nitrite is poisonous and carcinogenic which is generated by nitrate reducing bacteria during food fermentation process especially the initial stage. In this research, throughout the whole fermentation the nitrite concentration in *Lb.paracasei* HD1.7 fermented samples was extremely low (under 0.16 mg/kg, Fig. 5) and was even significantly lower than that in cabbage fermented by a mix starter *Lb.delbrueckii* IWQ and *Lb.paracasei* J21 (3.03 mg/kg) (Han *et al.* 2014). As was shown in Fig. 1, the addition of starter culture *Lb.paracasei* HD1.7 generated stronger acidity than the control. The faster and more produced lactate (Fig. 2) not only inhibits the growth of nitrate reducing bacteria but also contributes to nitrite decomposition. This has also been proved in other fermented vegetable food with LAB as starter cultures (Yan *et al.* 2008; Wang *et al.* 2010).

Amino acids are one kind of major flavor and nutritious substances in fermented cabbage. Because of the addition of *Lb.paracasei* HD1.7, seven out of seventeen amino acids as well as the total amino acids were significant higher (Table 1). This data generally suggested more nutritive value as the consequence of *Lb.paracasei* HD1.7. In contrast, Jung *et al.*'s research showed that starter *Leu.mesenteroides* did not provide significant differences in nutritive value in terms of amino acids (Jung *et al.* 2012).

Conclusions

The main conclusion to be drawn from the results of the research is that the addition of *Lb.paracasei* HD1.7, an isolate from Chinese fermented cabbage and producer of bacteriocin, as a starter culture makes the cabbage fermentation more nutritious and preferred by consumers. Compared to spontaneously fermented cabbage, *Lb.paracasei* HD1.7 fermented samples had higher content of lactate, vitamin C, mannitol and seven amino acids, lower content of nitrite as well as more satisfying sensory flavor. This research suggested strain *Lb.paracasei* HD1.7 an application potential in industrially producing fermented cabbage or other fresh vegetables with better characteristics and more nutritive values.

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