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Plant-based fermentation: Study of foodborne pathogens in spontaneous fermented vegetables

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Abstract: The aim of this study was to investigate the fate of Salmonella in the spontaneous carrots and cabbage fermentation. In order to achieve this purpose, it inoculated salmonella in the vegetables with and without adding of mixed starter-cultures. Then during the short term cultivation, the fermentation could be reproducible. For the carrots, after 7 days' fermentation, the population of salmonella maintained or had a little increase compared with the initial number and this number would have a reduction when vegetables were put into the refrigerator for 2 weeks. As for the cabbages, they had an obviously reduction in the amount of salmonella and there were no colony could be observed after 2 weeks' storage in refrigerator. In addition, the starter cultures can effectively suppress the growth of salmonella. Moreover it is necessary for spontaneous fermentation to have a scientific risk assessment to prevent the occurrence of such accidents.

1. Introduction

Vegetables play an important role in human diet for a long time, they can be served as fresh, minimally processed, sterilized or cooked by boiling or microwave. Thinking about these minimally treating methods may not have huge changes in vegetables, so the shelf-life is really short[1]. In that case, people working on the spontaneous fermented vegetables to prolong the shelf-life and maintain the healthy contents.

Normally the spontaneous fermentation is happened by the association between various naturally-occurring bacteria and raw materials. The vegetables will be put into the brine water treated with proper temperature and after a period they can be served as the appetizer[2]. As a well-known biotechnology in food preservation, fermented vegetables have a variety classification, such as Korean kimchi, Chinese Pao Cai and sauerkraut.

And based on the major products, fermentation can be classified into several processes: lactic acid fermentation, alcoholic fermentation, acetic acid fermentation, alkaline fermentation and amino acid fermentation[3]. Considering the fermented vegetables contain enough amount of essential nutrients, vitamins, minerals, antioxidants, fiber and during the lactic acid fermentation process, these strains of fermentative lactic acid bacteria which belong to species of genus Lactobacillus can help promote health, so this experiment preferred to take this method to perform the fermentation[4,5].

The fermented vegetables chosen were carrots and cabbages. Due to their fresh flavor, antioxidants, rich in vitamin and minerals, carrots were being consumed increasingly [6]. Moreover regarding to the health-promoting properties, sauerkraut which contained high antioxidant and anti-inflammatory compounds was the major dietary ingredient in Europe[7,8]. So in this experiment, these two typical vegetables were used for fermentation.

Through the process of adding salted water to fresh vegetables, the lactic acid bacteria which normally showed on the surface of food are allowed to multiply and flourish by offering an acidic inner environment to prevent spoilage. Also make sure all the fresh components submerged under the brine water can create an anaerobic environment which is really suitable for the growth of lactic acid bacteria. Thinking from another angle, this means that some potentially harmful or pathogenic bacteria cannot easily survive. Also some previous vegetable fermentations have used starter cultures resulted in improving the sensory characteristics, prolonging shelf-life and predominating over the native microorganisms present in raw materials[9].

Due to the aim of extension the shelf life, it is well known that large amount of innate background microflora could form into a pretty safe microbial environment which may suppress the growth of other contaminating bacteria in some degree. Indeed, the major inhibition spoilages are caused by lactic acid bacteria[10]. However, as the most common foodborne bacteria, salmonella have the ability to adapt the severe environment and survive[11].

Salmonella are common-seen Gram-negative rod-shaped bacteria which are important pathogen for both humans and animals[12]. The majority pathway for living bodies is through consuming the polluted water which may shred the bacteria into the feces and then humans and animals could infect the disease[12,13]. Since there's a high possibility to infect this bacteria, it's useful to figure out how salmonella survive among the fermented vegetables. And observe the changes of salmonella when they are in the fermented situation.

So in this experiment, the aim was to simulate the situation when homemade spontaneous fermented vegetables with and without starter cultures were invaded by salmonella bacteria and observe the fate of foodborne pathogen in fermented vegetable products. Moreover, through the observing, it can conclude the discipline and assist relevant risk assessment. Then trigger further research in this area to deeply explain the mechanisms.

2. Materials and methods

2.1 Chosen for bacterial strains

The bacteria used were Salmonella Montevideo and Salmonella Thompson688. And the starter-cultures were L fermentum pcc, L plantarum ML Prime, and L plantarum 299v. All of them were stored in the freezer at -20℃.

2.2 Pickle preparation and sampling

All the carrots were peeled and cut into pieces. Then all the sections were submerged under the brine water (5% w/v NaCl) and evenly poured into jars (carrots:solution=1:1). After inoculating, these jars were left for fermentation at 20℃.

2.3 Assessment of quality parameters

The whole process of fermentation was assessed by measuring physicochemical parameters and microbiological parameters. According to the data collected, the tendency and changes of the bacteria situation can be observed. And by analyzing the data, the results can be recorded and

concluded.

2.3.1. Physicochemical analyses

In this section, the physicochemical analyses included measuring pH value and the total titratable acidity (TTA).

And for measuring the TTA, transfer the sample solution to a flask or a beaker. Add 2-3 drops of phenolphthalein indicator and stir well. Then rapidly titrate the contents with 0.1 M NaOH solution, continually add alkali drop by drop and stir the contents until change to pink color. Record the final burette reading. And the TTA was expressed in % lactic acid.

$$\text{Lactic acid } \% = \frac{c(\text{NaOH}) * V(\text{NaOH})}{\text{Volume of sample}} * 100$$

2.3.2. Microbiological analyses

In this section, the microbiological analyses included total aerobic mesophilic count, lactic acid bacteria count and the inoculated pathogen count. The homogenized solution would be diluted by proteose peptones water (PPS: 0.9%NaCl 0.1% peptone). Spreading 0.1 mL of the diluted sample to the surface of the media. Then the plates were prepared. After culturing, count the amount of bacteria and figure out the salmonella situation.

2.4 Data analysis

All the experiments were performed in triplicate. As for data processing, calculating the average value, known as the average, provides a quick snapshot of all data. In addition to the mean, only the standard deviation was measured to display the distribution of the data around the mean.

3. Results

3.1 First trial

Prepared all the materials in advance, such as agars, autoclaved tubes, PBS and PPS for dilution. Then activated the bacteria: Salmonella Montevideo and Salmonella Thompson688. After activating, the fresh bacteria needed to be purified with phosphate buffer solution (PBS). Centrifuge at 6000 rpm for 10 minutes for three times. Then the final solution can be diluted or inoculated and the bacteria should be cocktailed before inoculation.

After finishing all the procedure with the bacteria, they can be inoculated into the vegetables. For the first experiment, chosen 4.5kg of carrots and divided into 9 jars with brine water. These 9 jars were divided into three groups: control level group (no inoculation), high level group (inoculated around 5.0 log CFU/mL), and low-level group (inoculated around 3.0 log CFU/mL). After inoculating, these jars should be left for fermentation at 20℃. Samples were taken at the unfermented day and the 7th day. The data were recorded as follows:

Table 1: Results of low-dose inoculated group

	Day 0	Day 7
Salmonella pathogen count (log cfu/ml)	2.620 ±0.046	2.780 ±0.350
Total aerobic mesophilic count (log cfu/ml)	4.130 ±0.069	7.880 ±0.120
Lactic acid bacteria count (log cfu/ml)	2.413 ±0.045	7.750 ±0.180
PH	5.647 ±0.060	3.340 ±0.030

Trail 1. The changed data of total aerobic mesophilic count (PCA), lactic acid bacteria (MRS)

count, Salmonella pathogen count (XLD), pH at low, high and control levels between day 0 (unfermented day) and day 7 of the first trail[A]， as shown in Table 1,2,3.

Table 2: Results of high-dose inoculated group.

	Day 0	Day 7
Salmonella pathogen count (log cfu/ml)	5.460 ±0.053	4.550 ±0.130
Total aerobic mesophilic count (log cfu/ml)	5.653 ±0.136	7.690 ±0.040
Lactic acid bacteria count (log cfu/ml)	2.297 ±0.170	7.580 ±0.200
PH	5.553 ±0.015	3.230 ±0.040

Table 3: Results of control (no inoculated) group

	Day 0	Day 7
Salmonella pathogen count (log cfu/ml)	0	0
Total aerobic mesophilic count (log cfu/ml)	3.72 ±0.061	7.55 ±0.10
Lactic acid bacteria count (log cfu/ml)	1.93 ±0.151	7.60 ±0.19
PH	5.68 ±0.026	3.35 ±0.06

According to the trail 1, during 7 days' fermentation, there were some changes among the physicochemical and microbiological parameters. For control group, without inoculation, there was no colony showed on XLD agars. For the other two groups, the number of salmonella had a little increase from 2.62 to 2.78 log cfu/ml in the low level group, while there was a decrease from 5.46 to 4.55 log cfu/ml in the high level group. And with the reduction of PH, the amount of lactic acid bacteria increased by 1.5 times reached up to 7 log cfu/ml among the three groups regardless the initial level.

3.2 Second trial

For the second trail, on the basis of the first trail, just added the starter cultures in the carrots. And there would be four groups: control group (no inoculation), low level of salmonella group (3 log CFU/mL), starter-cultures group, low level of salmonella and starter-cultures group. The salmonella was prepared as usual while the starter-cultures: L fermentum pcc, L plantarum ML Prime and L plantarum 299v would be transferred once by using the MRS broth.

In order to verify the existence of bacteria, this study prepared an enrichment by mixing 20ml brine water with buffer peptone water and stored in the incubator at 37℃. All the data were in the below tables:

Trail 2 The changed data of total aerobic mesophilic count (PCA), lactic acid bacteria (MRS) count, Salmonella pathogen count (XLD), pH at control, low, starter-culture, low+starter-culture groups among day 0 (unfermented day), day 7 and after two weeks which were put in the refrigerator of the second trail[A], as shown in Table 4,5,6,7

Table 4: Results of control groups.

	Day 0	Day 7	2 weeks
Salmonella pathogen count (log cfu/ml)	0	0	0
Total aerobic mesophilic count (log cfu/ml)	4.897 ±0.061	7.180 ±0.120	7.530 ±0.110
Lactic acid bacteria count (log cfu/ml)	2.327 ±0.057	7.240 ±0.180	7.950 ±0.150
PH	5.610 ±0.010	4.080 ±0.040	4.130 ±0.020

Table 5: Results of low-dose inoculated groups

	Day 0	Day 7	2 weeks
Salmonella pathogen count (log cfu/ml)	3.147 ±0.012	3.280 ±0.030	1.010 ±0.098
Total aerobic mesophilic count (log cfu/ml)	3.910 ±0.040	6.170 ±0.280	6.840 ±0.036
Lactic acid bacteria count (log cfu/ml)	2.957 ±0.067	6.080 ±0.540	6.973 ±0.081
PH	5.720 ±0.010	3.980 ±0.020	4.263 ±0.006

Table 6: Results of low-dose salmonella and starter-cultures inoculated groups

	Day 0	Day 7	2 weeks
Salmonella pathogen count (log cfu/ml)	3.150 ±0.087	0.900 ±0.17	0
Total aerobic mesophilic count (log cfu/ml)	5.480 ±0.185	7.750 ±0.02	7.730 ±0.08
Lactic acid bacteria count (log cfu/ml)	5.670 ±0.053	7.680 ±0.13	7.820 ±0.08
PH	5.673 ±0.015	3.870 ±0.01	3.760

Table 7: Results of starter- cultures inoculated groups

	Day 0	Day 7	2 weeks
Salmonella pathogen count (log cfu/ml)	0	0	0
Total aerobic mesophilic count (log cfu/ml)	5.717 ±0.047	8.210 ±0.090	7.507 ±0.254
Lactic acid bacteria count (log cfu/ml)	5.757 ±0.081	8.190 ±0.190	7.667 ±0.091
PH	5.753 ±0.025	3.850 ±0.010	3.803 ±0.006

[A] Data are mean ±SD.

Compared with the first trail, the only change of the second one was adding the starter- cultures. There were no big differences in control and low groups. As for the low level group, the population of salmonella had a small increase from 3.15 to 3.28 log cfu/ml. And as for the group mixed with salmonella and starter-cultures showed an obviously reduction from 3.15 to 0.9 log cfu/ml. During 7 days' fermentation, the total amount of bacteria almost had 2 times increasing. And the number of lactic acid bacteria (LAB) had the same change like the first trail, when the PH changed from 5 to 3, the amount of LAB increased more than 2 times. Also it was worth mentioning that the two groups adding with starter-cultures had a pretty high amount of LAB in initial, respectively.

Then after 7th measurement, we put the samples in the refrigerator for 2 weeks and then took out for measuring. The amount of salmonella in the low group decreased from 3 log CFU/ml to around 1 log CFU/ml. Meanwhile, there was no colony could be observed in the low+starter-cultures group, even with the enrichment test. And for control and low groups, the total and LAB amount both had a small increase. While for the group only added with starter-cultures showed a slightly decrease on total number of bacteria and the amount of LAB. As for the group with salmonella and starter-cultures, it reported that the total number of bacteria was almost the same as the data of 7th day and the population of LAB had a little rise.

3.3 Third trial

The preparation procedure of the bacteria was the same as the second trial. One different thing was the type of vegetables, we changed the carrots into cabbages and used dry fermentation method. Firstly washed the cabbages (2kg) and cut them into pieces. Then mixed the cabbage with salt (3%) layer by layer and divided into 4 jars (Control, no inoculate; Low, around 3 log CFU/g; Starter-cultures; Low-dose salmonella+Starter- cultures inoculated). Also the samples would be measure at the unfermented day, the 7th day and 2 weeks later in the refrigerator.

Table 8: Results of control group.

	Day 0	Day 7	2 weeks
Salmonella pathogen count (log cfu/g)	0	0	0
Total aerobic mesophilic count (log cfu/g)	3.837 ±0.206	6.540 ±0.030	5.060 ±0.104
Lactic acid bacteria count (log cfu/g)	1.160 ±0.151	6.530 ±0.100	5.050
TTA(%)	0.1 ±2E-17	0.550 ±0.010	0.470 ±0.001

Table 9: Results of salmonella inoculated group

	Day 0	Day 7	2 weeks
Salmonella pathogen count (log cfu/g)	2.533 ±0.112	0.360 ±0.100	NO
Total aerobic mesophilic count (log cfu/g)	2.937 ±0.072	7.290 ±0.100	5.493 ±0.101
Lactic acid bacteria count (log cfu/g)	2.423 ±0.071	7.410 ±0.120	5.573 ±0.192
TTA(%)	0.1 ±2E-17	0.550 ±0.010	0.530 ±0.001

Table 10: Results of starter-cultures inoculated group

	Day 0	Day 7	2 weeks
Salmonella pathogen count (log cfu/g)	0	0	0
Total aerobic mesophilic count (log cfu/g)	5.307 ±0.076	7.420 ±0.020	6.033 ±0.172
Lactic acid bacteria count (log cfu/g)	5.400 ±0.078	7.370 ±0.030	5.857 ±0.102
TTA(%)	0.1 ±2E-17	0.500 ±0.010	0.495 ±0.100

Table 11: Results of starter-cultures and salmonella inoculated group

	Day 0	Day 7	2 weeks
Salmonella pathogen count (log cfu/g)	2.633 ±0.064	0	NO
Total aerobic mesophilic count (log cfu/g)	5.140 ±0.187	7.730 ±0.010	5.663 ±0.164
Lactic acid bacteria count (log cfu/g)	5.323 ±0.049	7.720 ±0.010	5
TTA(%)	0.1 ±2E-17	0.580 ±0.010	0.530 ±0.010

Trail 3. Changes of total aerobic mesophilic count (PCA), lactic acid bacteria (MRS) count, Salmonella pathogen count (XLD), pH and TTA at control, low, starter-culture, starter-culture+low levels among day 0 (unfermented day), day 7 and after 2 weeks which were put in the refrigerator of the third trail[A], as shown in Table 8,9,10,11.

[A] Data are mean ±SD.

In 7th day's measurement, there was nothing on the XLD agar of starter-cultures+low- dose of salmonella inoculated group. So we took out the enrichment solution and streaked on the XLD plate. After 24h, there was still nothing on the XLD. The same situation happened in the samples which were put in the refrigerator for 2 weeks.

For the group with salmonella, the amount on the XLD were decreased obviously from 2.5 to 0.3 log cfu/g and for the group with salmonella and starter cultures had an even more obviously reduction from 2.6 to 0 log cfu/g. And other parameters were followed the similar trend like first two experiments.

4. Discussion

4.1 The process of spontaneous fermented vegetables

Just as the definition, the spontaneous fermentation process was about allowing natural bacteria originally present on the vegetables to start the fermentation. Therefore, there are no microorganisms added for this process to start. However, it is important to offer the optimal environment to promote bacterial growth. In this study, the first experiment was totally followed the spontaneous fermentation procedure without adding any starter cultures, which was like a control group.

Then talking about the whole chemical and microbial changes, just take the sauerkraut fermentation as an example, which had four different phases normally[14]. In the first phase, the vegetables were cut and put in the brine water. Through the respiration of plant cell, CO2 and heat were produced. Then in the second phase, with the increasing of the acid concentration, the microaerophylic lactic acid gradually took the lead position. Next in the third phase, there would be the homofermentation with a little increasing of the lactic acid. Finally for the fourth phase, the PH was reduced to around 3. During all these phases, the lactic acid fermentation was associated with the fermentation time, salt concentration, culture temperature and the types of vegetables.

And in order to provide right environment for fermentation, this study used microbiological methods, such as sterilization the medium and heating by the autoclave machine. In previous studies [15,16], they thought heating can destroy vitamins and provoke Maillard reactions which can reducing the free amino acids.

4.2 Interpret and describe the findings

For the first trail, the subjects were carrots and were divided into control group and salmonella groups. Next for the second trail, on the basis of previous one, added the starter-cultures to observe whether they can influence the survival of salmonella. We also subjoined a measurement to detect the population of salmonella when the carrots had been put in the refrigerator for two weeks. Then as for the third trail, we changed the fermented vegetables from carrots into the cabbages and other operation processes were the same as the second one.

Among the three experiments, we can clearly observe the discipline. During the 7 days' fermentation, the PH of the fermented vegetables turned from neutral to acidic accompanying with the increase of the lactic acid bacteria. And the population of salmonella almost maintained the initial level or had a little bit rising and then had an obviously reduction when they cultured in the

refrigerator which means that the low temperature had the stress pressure on the salmonella. Moreover, with the adding of starter-cultures, the groups showed a huge decreasing in 7 days' fermentation and there was no colony can be observed in two weeks from the refrigerator which indicated that environment with starter-cultures had a negative effect on the salmonella bacteria. According to the information above, we can preliminary conclude that the acidic environment and the addition of starter-culture can suppress the growth of salmonella to some extent.

Moreover, we can observe that different types of vegetables had different levels of stress for salmonella bacteria, in this study, it showed the cabbage fermentation had a more obviously reduction in salmonella compared with carrots fermentation. For the carrots, they can be just cut into the brine water. While the cabbage, thinking of the mildew problems, we preferred to choose the dry fermentation without any extra water. When we measure the samples, for the carrots' groups, we just took the brine water with was mixed with salmonella well. And for the cabbage, we needed to pick some slices and soak them in the peptone water which may be cannot present totally the real amount. Also in the first two experiments, the salmonella we chose were just activated from the freezer, while the last one was selected from the streaked TSA agar which may cause the less quantity. Moreover, there were more cabbage fermentation than carrots fermentation, maybe cabbages had more nutrients compared with carrots for fermentation. As for the salmonella, the salmonella Thompson strain was isolated from cilantro, while the salmonella Montevideo was isolated from tomato outbreak. They were both obtained from Dr Maria Brandl (U.S. Department of Agriculture, Agricultural Research Service, Albany, CA, United States).

4.3 The effect of starter-culture

The mixed starter cultures involved in the last two experiments were listed in the Table 12. With the treatment of starter cultures, there were usually positive effects on vegetables[17].

Compared with natural fermentation, using fermentation agents can shorten fermentation time and accelerate acidification. It also ferments, and vegetables rely on the initial bacterial raw materials, without strict control, sometimes they may obtain some undesirable final products.[18]

Although mostly the lactic acid bacteria starters are used in milk, meat and baked good fermentations, still have a few cultures which can be used for vegetable fermentations and the most typical one is the lactobacillus plantarum. And also another study had reported that the lactobacillus fermentum can be isolated from the Chinese Paocai[19]. So in conclusion, these three types of starter cultures were quite suitable to apply on the vegetable fermentations.

Table 12: Information of the starter-cultures involved in the study

Types of probiotics	Source	Used as	References
Lactobacillus fermentum pcc	Chr. Hansen	Probiotic, obligate heterofermenter	West et al, 2011
Lactobacillus plantarum ML prime	Lallemand	Wine making for malolactic fermentation, facultative heterofermenter	Brizuela et al, 2018
Lactobacillus plantarum 299V	Probi	Probiotic, facultative heterofermenter	Świeca et al, 2018

4.4 Prevention measurements

Firstly, the important thing was the choice of fermented vegetables. In this study, it is clearly that the cabbage had more favorable effects on fermentation than the carrots. So it is also important to

find a suitable vegetable which the initial bacterial were strong enough against the exotic bacteria.

Secondly, making sure all the instruments of fermentation are hygienic and sterile, including the jars, stones, knives. All the agars were autoclaved and the operation tools like loops, streaks and tubes were all sterilized. In this case, we can make sure there are no other unwanted bacteria involved in the fermentation system.

Thirdly, creating a suitable environment for fermentation. Before a spontaneous fermentation, we should settle down the salt concentration, PH and decide the culturing temperature which are good for fermentation and are harmful for the growth of bacteria. As mentioned before, with the decreasing of PH, there is the reduction of the Salmonella, so it prefers to produce a quite acidic environment.

Fourthly, as a culture of microorganism, starter cultures are one of the efficiency ways to meet the food quality and produce a low PH to prevent spoilage. Of course, the chosen of starter cultures is according to the types of vegetables.

4.5 Strength

The first strength for this study was its reproducibility. The fermentation methods can be applied into other experiments.

Also there were something consistently among three experiments. The first trail was kind like a big control group showing the spontaneous fermentation involved salmonella. And as for the second one, it maintained the basic group with salmonella and added a new group with the starter culture to detect the effects of starter cultures on changes of salmonella. Then the last one, contained all the group like previous one by using a new kind of vegetable. So it is kind of a progressive experiment, each one maintained some parts of previous one and create a new condition.

5. Conclusion

Summarizing the results of this study, we can draw the conclusion that the salmonella added in the spontaneous fermented vegetables can be survived in 7 days' observation, but they were suppressed by the increased acidity, reduced temperature and the adding of suitable starter cultures. Also this study want to provide some safety advice to help fermented food products stay away from the invasion of foodborne bacteria.

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