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Preservative adulteration in soybean products signaling severe food safety problems has been improved by good hygienic practice

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Abstract

As a rule, the Tofu manufactories are apt to be polluted and contaminated with a huge population of microbes, which in turn may contaminate the product Tofu and related product. In order to extend the shelf life of Tofus, some illegal prohibited preservative had been abused in Tofus. During the year 2006 to 2008, the illegal cases of Tofu products in Taiwan reached 233 cases. The illegally used preservatives involved hydrogen peroxide, salicylic acid, and dehydroacetic acid. In 2010 we organized a food safety team under the 'Post-Market Training and Supervising Project' (PTSP) and executed the Good Hygienic Practice (PTSP).

Before the finishing of GHP training, among the products examined, a 33.3% to 66.6 % of the products were highly abused with salicylic acid and dehydroacetic acid during the year 2010-2014. Others (33.3% of the products) were adulterated with overdose of *p*-hydrxybenzoate. The absolute number of manufacturers that had been abusing the use of preservatives in fact was substantially reduced from 233 cases before GHP training (during 2006 to 2008) to merely 16 cases after GHP training (during the year 2010 to 2014) implicating the effectiveness of the GHP training. At the same time, the sanitation ranks of the environment *in situ* the Tofu manufacturing sites and the Tofu products were greatly improved, most of the product Tofus showed a sanitation rank of log cfu/g as low as 1-2.

Keywords: food safety, soybean products, PTSP, PTSP

Introduction

Soybeans are one o-f the most important cash crop in the United States, and they contribute more protein and fat to our food economy than any other single source (Liener, 1978) [32]. Soybean protein is unique among plant proteins because of its relative high biological value (Schroder *et al*., 1973) [42]. Like many soya foods, Tofu originated in China. Legend has it that it was discovered about 2000 years ago by a Chinese cook who accidentally curdled soy milk when he added nigari seaweed. Introduced into Japan in the eighth century, Tofu was originally called *okabe*. Its modern name did not come into use until 1400. According to industry statistics gathered by Shurtleff and Aoyagi (1983) [43] of the Soyfoods Center in California, Tofu has been made commercially by Asian immigrants in the United States since 1904. By the 1960s, interest in healthy eating brought Tofu to Western nations. Since that time, countless research has demonstrated the many benefits that soya and tofu can provide (Lewin. 2014). For processing, various methods have been developed to make soybean as palatable as possible. Three types of soybean curd are produced in the Orient: soft Tofu, hard Tofu, and dried Tofu. In addition, many different products related to Tofu, such as Tou-chi (bean-chicken), savory toukan, chien-chang, yuba, fried Tofu and fermented Tofu also are made (Tsai *et al*., 1981) [48]. Soft tofu, with water content as high as 87-90 % and a smooth, fragile curd, is especially popular in Japan (Anonymous., 1954; Tsai *et al*., 1981) [3, 48].

However its safety and sanitation problems are emerging, in particular the processing of Tofu and related products. The tofu product safety and sanitation have raised many problems in all Tofu-making countries including China (Apple News, 2007), Korea (Wang *et al*., 2009) [52], Taiwan (http://www.channelnewsasia.com/news/asiapacific/taiwan-recalls-tainted/1535406.html),

Japan (Inagari, 2014; Ledbetter, 2014) [29], and the United States (Anderson, 2015) [2]. A recent report indicates McDonald's just might have become a tofu lover's fast food delight (Ledbetter, 2014) [29]. Attributed to the fallout from a Chinese food supplier (though some are claiming the move was planned well in advance) sending rotten meat to restaurant chains, McDonald's in Japan decided to play it safe and chicken-free by turning to tofu (Ledbetter, 2014) [29].

Recently, FDA (USA), finding horric sanitation conditions, ordered the shut- down of a Sacramento Tofu producer (Anderson, 2015) [2]. According to a federal complaint, inspectors with the Food and Drug Administration found violations at the company's production facility in 2003, 2005, 2008, 2010, 2011 and 2014. With the most recent inspection, in the summer of 2014, the inspectors found even worse conditions (Anderson, 2015) [2]. Inspectors also found food safety practices that are not acceptable, including splashing water from the floor into production areas and multiple instances of cross-contamination of food products with employees' dirty hands (Anderson, 2015) [2].

In Taiwan, Tofu products have owned a huge market. The commercialized soybean Tofu products can be roughly divided into hard Tofu curd, soft Tofu curd, Dried Tofu curd and soybean protein sheets (To-Bao), according to their degree of dryness or moisture content, the amount of coagulant used (usually gypsum or calcium sulfate), the coagulation temperature, and the stirring time (Detection monthly, 144).

The regular additives ever used in the TOFU manufacturing may include 1) coloring agents, 2) coagulating agents, 3) deformers, 4) emulsifiers, 5) preservatives, and 6) bacteriocides.

The problems currently existing with the Tofu manufacturing involve 1) the microbial deterioration caused by the contamination from processing, packaging, and storage (Kovats *et al*., 1984; Rehberger and others 1984; Ashenafi, 1994; Kim and Son, 2004); 2) the over dosing of additives; and 3) the use of prohibited additives.

According Lee (2009), tremendous under-qualified cases were committed to the use of the prohibited preservatives, approaching chronologically up to 11%, 6%, and 11% respectively in the year 2006-2008, mostly concerning the over abuse of the preservatives.

Materials and methods

Facing such a food safety and sanitation problem, we were determined to establish 'the Post-Market Training and Supervising Project'.

Based on the protocol and regulation issued by the Good Hygienic Practice (GHP), the good restaurant sanitation evaluation sheet, and the Hazard Analysis & Critical Control Points (HACCP), we took the first step to organize the HACCP team, assigning each individual responsibility and then proceeded repeatedly with monitoring, training, re-education, records filling, resampling with re-examination (Table S1 in Supplement). The membership involved the FAD officers, the industrial experts, and the scholars from the academic agents. And we arranged two strategies: one was the movement of GHP under 'the Post-Market Training and Supervising Project', and the other the simultaneous 'Inspection of the Tofu and related products'.

Post-Market Training and Supervising Project

As reported, the soybean derived products have been illegally added with some prohibited food preservatives like sorbic acid, benzoic acid dehydroacetic acid (DHAA) and hydrogen peroxide (HPO), which in reality already have severely abused the safety of foods and potentially threatened the public life and health. As one of the authorized Bureaus of Food Sanitation and Safety, we decided to selected 19 manufacturers that have been found involved in the food safety problems to proceed the 'Post-Market Training and Supervising Project (PTSP)'. Briefly, this PTSP actually was executed in three steps:

Step 1: We established the Good Hygienic Practice (GHP). By following GHP, we designed a record sheet of GHP (Table S1, Table S2 in Supplement) and asked the Tofu-manufacturers to follow the guidance. In which we have listed 25 items involving three main aspects, i.e. 1) employees' sanitation, 2) environmental sanitation, and 3) management of food materials and additives (see Table S1 and Table S2 in Supplement).

Step 2: Before any action was taken, we visited at their manufacturing sites giving them a serial training courses, guiding them to obey the GHP and the regulations of 'Environmental Sanitation *in situ* the Manufacturing Sites' as well as the 'Disinfection of the Working employees, aiming to strengthen their knowledge about the 'Food safety with Food Additives'.

Step 3: In addition, we sampled frequently the Tofu products *in situ* to examine the microbial contamination as well the amount of violating preservatives.

The PTSP (2010-2014) was executed by our Bureau of Sanitation and Food Safety and brought into action since 2010. In this operation, we sampled a number of the on-shelf Tofu products and examined the presence of some prohibited preservatives (Fig. 1). If any, the crimes will be subjected to prosecution with fine by the authorized Bureau of Food Safety and Sanitation by the reason 'being violating 'The regulations of Food Safety and Control'. In such a way, we expected the guilty case can be terminated.

The training protocol was designed as shown in the protocol sheet, which actually included three aspects, i.e. 1) the sanitation and health condition of the staffs; 2) the environmental sanitation; and 3) the management of materials and food additives; which was further divided into 25 subitems as shown, which in reality was designed on purpose as a food safety and management chart applicable to Tofu industries.

Supplement Tables

Table S1: Example of HACCP plan (by Kanduri and Eckhardt, 2002, depicted from Fishing News Books, a division of Blackwell Publishing).

An Illustrated Example of a HACCP Plan – Processing Cooked Shrimp* (See Chapter 3)

ASSEMBLING A HACCP TEAM AND ASSIGNING RESPONSIBILITIES (FORM #1)

Responsibility: Hazard identification
Name of the designee:
1.
2.
3.

Responsibility: CCPs determination
Name of the designee:
1.
2.
3.

Responsibility: Monitoring CCPs
Name of the designee:
1.
2.
3.

Responsibility: Verification of operations at CCPs
Name of the designee:
1.
2.
3.

Sample testing and verification:
Name of the designee:
1.
2.

Kanduri L. and Eckhardt R.A. 2002. Food Safety in Shrimp Processing

Example taken from Canadian Food Inspection Agency QMP plan.
Example taken from Canadian Food Inspection Agency QMP plan

Table S2: Designed 'good hygienic practices code chart' showing guidance items for the Tofu and related soybean product manufacturers.

工作人員衛生(1)	
1-1	每年應主動健康檢查乙次
1-2	設有更衣室及個人存放衣物之箱櫃
1-3	作業人員，工作時應穿戴整潔之工作衣帽(鞋)，必要時應戴口罩
1-4	作業人員雙手保持乾淨，不留過長指甲、塗指甲油及配戴飾物
1-5	工作中不得有吸菸、嚼檳榔等可能汙染食物之行為
1-6	手部受傷的員工嚴禁直接接觸熟食物或從事包裝工作
作業環境整潔(2)	
2-1	牆壁、支柱、天花板、燈飾、紗門、紗窗等應保持清潔，不可有藏汙納垢、侵蝕等情形
2-2	維持暢通之排水系統，地板須清潔，不得有積水、濕滑等現象
2-3	應有良好通風及排氣設備
2-4	依清潔度要求不同之場所，應加以有效區隔及管理
2-5	工作場所照明光線應達到一百米燭光以上，工作檯面或調理台應保持兩百米燭光以上
2-6	進出工作場所應實施有效之病媒防治措施
2-7	食物不得直接放置地面，若以器皿裝盛放置地面應加蓋

2-8	冷藏冷凍庫中存放之物品應覆蓋或包裝好，擺放整齊
2-9	器具洗滌應使用合法食品清潔劑沖洗、殺菌消毒並排放整齊
2-10	垃圾、廢棄物應妥善處理，垃圾桶應加蓋
2-11	工作場所內不得飼養牲畜
2-12	廁所需與調理食物場所隔離，經常保持乾淨，有妥善之洗手設備。張貼{如廁後應洗手}標語及洗手步驟
	物料及食品添加物管理(3)
3-1	原材料來源資料建檔，倉儲過程中應定期檢查，並確實記錄
3-2	倉庫應設有效防止病媒(蟑螂、老鼠等)侵入之設施
3-3	倉庫應設棧板，離地離牆各五公分以上，並保持清潔定期清掃，良好通風及良好溫濕度控制
3-4	原材料、半成品及成品分區儲存管理
3-5	原物料之使用應依先進先用之原則，避免混雜使用或過期使用
3-6	架上食品物料陳列整齊，定期清潔，並不得過期
3-7	食品添加物應設專櫃存放，由專人負責管理，並以專冊登錄使用之種類、食品添加許可字號、進貨量、使用量及存量

Table S3: The good hygienic practice record of soybean products manufacturer.

南投縣政府衛生局豆麵業者食品良好衛生輔導記錄表 年 月 日合法□是 □否		
地點	廠商名稱： 負責人姓名：	廠商地址： 衛生管理人： 電話： 營利事業登記字號：
工作人員衛生	每年應主動辦理健康檢查乙次。	
	設有更衣室及個人存放衣物之箱櫃。	
	作業人員，工作時應穿戴整潔之工作衣帽(鞋)，必要時應戴口罩。	
	作業人員雙手保持乾淨，不留過長指甲、塗指甲油及配戴飾物。	
	工作中不得有吸菸、嚼檳榔等可能污染食品之行為。	
	手部受傷的員工嚴禁直接接觸熟食物或從事包裝工作。	
作業環境整潔	牆壁、支柱、天花板、燈飾、紗門、紗窗等應保持清潔，不可有藏污納垢、侵蝕等情形。	
	維持暢通之排水系統，地板需清潔，不得有積水、濕滑現象。	
	應有良好通風及排氣設備。	
	依清潔度要求不同之場所，應加以有效區隔及管理。	
	工作場所照明光線應達到一百米燭光以上，工作台面或調理台面應保持二百米燭光以上。	
	進出工作場所應實施有效之病媒防治措施。	
	食物不得直接放置地面，若以器皿裝盛放置地面應加蓋。	
	冷藏冷凍庫中存放之物品應覆蓋或包裝好，擺放整齊。	
	器具洗滌應使用合法食品清潔劑沖洗、殺菌消毒並排放整齊。	
	垃圾、廢棄物應妥善處理，垃圾桶應加蓋。	
	工作場所內不得飼養牲畜。	
	廁所需與調理食物場所隔離，經常保持乾淨，有妥善之洗手設備。張貼「如廁後洗手」標語及洗手步驟□有 □無。	
物料及食品添加物管理	原材料來源資料建檔，倉儲過程中應定期檢查，並確實記錄。	
	倉庫應設有效防止病媒（蟑螂、老鼠等）侵入之設施。	
	倉庫應設棧板，離地離牆各五公分以上，並保持清潔定期清掃，良好通風及良好溫濕度控制。	
	原材料、半成品及成品分區貯存管理。	
	原物料之使用應依先進先用之原則，避免混雜使用或過期使用。	
	架上食品物料陳列整齊，定期清潔，並不得過期。	
	食品添加物應設專櫃貯放，由專人負責管理，並以專冊登錄使用之種類、食品添加許可字號、進貨量、使用量及存量等。	
其他	1.於明顯處所懸掛工作人員 專業證照□有 □無 2.防腐劑 取樣□有 □無 3.衛生檢測 取樣□有 □無	
簽名	衛生局人員	輔導人員簽名

Inspection of the Tofu and related products

The actual inspection by this project was started from April 2013 to December 2013. We selected 19 manufacturers from 13 counties in Taiwan. Counting by product spectrum, it covered in majority Tofu foods (14 items), dried soybean curd (5 items), soybean protein sheet rolls (3 items), and others (1 item), which comprised of 61% of Tofu manufacturers, 22% of dried bean curd makers, 13% of soybean protein sheets makers, and 4% of other soybean products (Fig. 1).

Fig. 2 indicates the process lines for manufacturing the soybean products originated from the soybean milk. Screened soybeans of top quality were rinsed with deionized water and submerged int water for 8-10 h, after dewatered 5-6 folds deionized water was added and the wetted and swollen soybeans were blended and boiled at 98-105ºC for 5-6 min. the soybean boiled mashes were subjected to filter press to obtain soybean milk. Which was processed independently in four processing lines to produce packed Tofu, conventional Tofu, died soybean curd, and soybean sheets and sheet rolls (Fig. 2).

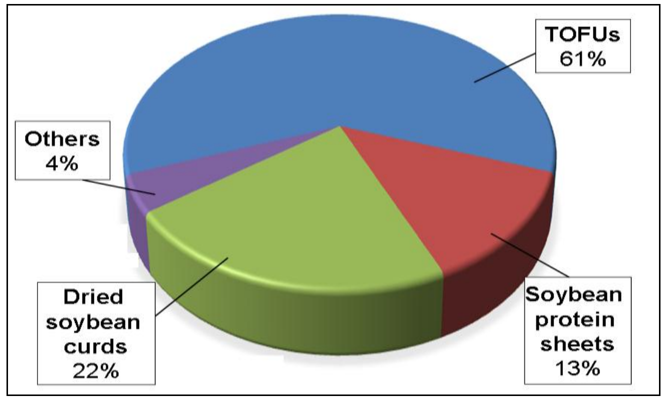

Fig 1: The distribution of the target Tofu-related products currently produced by the manufacturers under our GHP inspection project. GHP: good hygienic practice

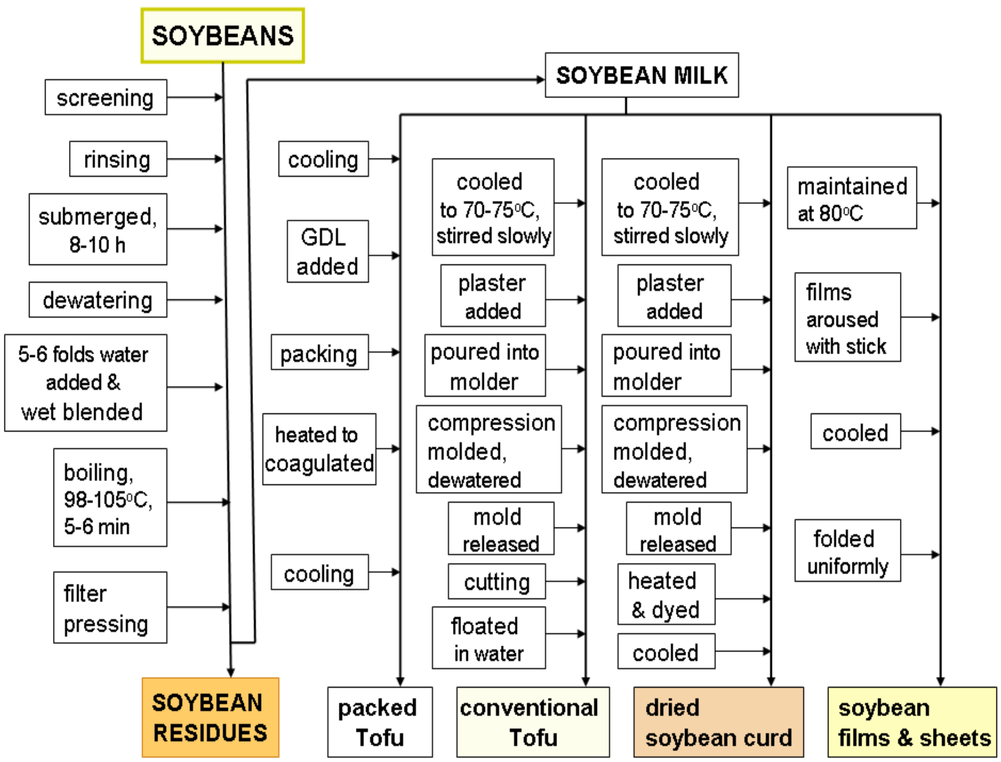

Fig 2: Flowchart for manufacturing different soybean products.
GDL: gluconate-δ- lactone.

Execution of PTSP-The beginning of GHP training

When starting with the GHP, we summoned the Tofu manufacturers, opening a serial lectures regarding the food safety and sanitation, leading a serial in situ training and pre-ranking, the latter included various sanitation rules, microbiological technology, and the knowledge dealing with food additives especially the food preservatives like formaldehyde, hydrogen peroxide, acidic preservatives, and esteric preservatives (Fig. 3). We designed an inspection chart, in which we were aiming three aspects of control: 1) the sanitation of the working staffs, 2) the environmental sanitation in situ the manufacturing sites, and 3) the management and control of materials and food additives (See Table S2 in Supplement). Such an inspection with re-education presented at least two times before and after the GHP.

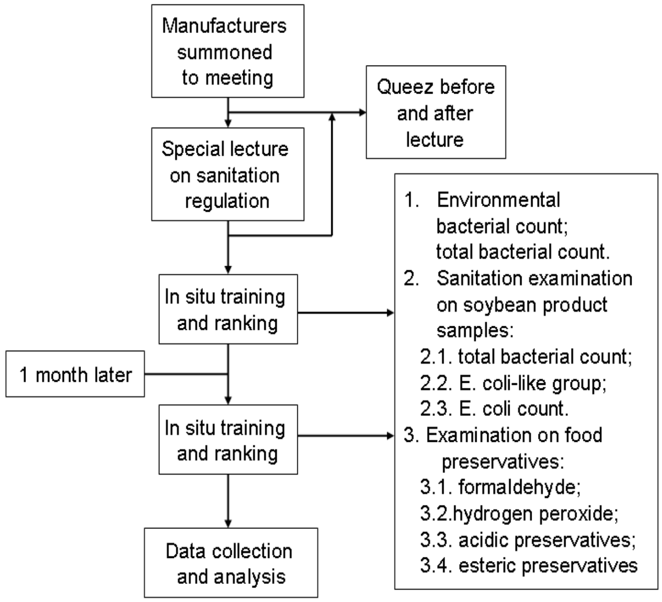

Fig 3: GHP supervising and training program as proposed by our Post-Market Training and Supervising Project (2010-2014). GHP: good hygienic practice

Evaluation and record of the outcome of GHP

In each local FDA subdivision, we designed an evaluation chart which gave a reliable evaluation and record of the outcome of GHP

Determination of food preservatives

Formaldehyde was determined according to the Test Method for Formaldehyde in Food issued by the Ministry of Food and Drug Administration (Taiwan) (FDAT) issued on 6 September, 2013, Document No. FDAT Food 1021950329). The determination for hydrogen peroxide was carried out by following the Method of Test for Hydrogen Peroxide in Foods (Document No. FDAT Food 1011900257, issued on 20 March, 2012). Acidic and esteric preservatives were examined according to the Method of Test for Preservatives in Foods, issued on 19 November, 2012 based on the Document No. FDAT Food 1011903320).

Inspection of the environmental sanitation

Similar protocol was used for examination of the environmental sanitation. The prepared PCA was exposed at the selected manufacturing sites, each site with 2 PCA, after uncovered and exposed to the atmosphere for 10 min, the cover was replaced and the PCA was inversed and incubated at 37ºC for 48±2h. The cfu/plate was obtained from the two PCA counting results.

Inspection of microbial contamination of Tofu products

The microbial examination was conducted by following the issue described in the Chinese National Standards (Taiwan) (CNS, Taiwan) No. CNS 10890 N6186 'Method of test for food microbiology - Test of standard plate count (Aerobic plate count) and 'The Examination Methods for Food Microorganisms" (issue No. 1011900543) announced by the Food and Drug Administration of The Ministry of Sanitation and Wealth (Taiwan) as of the date March 23, 2012, revised thereafter on September 6th, 2013 (issue No. 1021950329), i.e. the Plate Count Agar (PCA) method. PCA was prepared as follows: Agar powder with distilled water was melt at 55ºC. A portion of 12-15 mL of the melt agar was mixed with the appropriately diluted aliquot (1 mL), mixed well and immediately poured into the petri dish and left to cool to 45±1ºC. To prepare samples, the liquid sample was directly diluted with sterilized (121ºC, 15 min) saline in series (100×, 1000×, and 10000× etc.) to obtain an appropriate cell population. An aliquot (1 mL) of the diluted solution was incubated at 37ºC for 48±2h on PCA. For solid samples, to 25 g of the solid samples 225 mL of saline was added and blended in a sterilized (assigned as 10 fold dilution, 10×) dilution flask. The sample blend was then diluted serially to 100×, 1000×, and 10000× etc. An aliquot (1 mL) of the diluted solution was incubated at 37ºC for 48±2h on PCA and the cell population was counted from the colonies formed and the dilution factor. Sterilized saline was used as the blank. Duplicate experiment was conducted for each sample. The cfu/plate was counted and averaged from the two PCA counting results. The total bacterial count was calculated from the dilution folds and the amount of sample used.

E. coli like count in products

Preparation of the dilution agent: 8.5g NaCl was dissolved in 1000 mL distilled water and divided into several 250 mL bottles and sterilized at 121ºC for 15 min.

The detection of *E. coli* like bacilli was conducted according to the manufacturer's instruction. To make the ten-fold diluted sample solution, the following protocol was conducted: For the solid sample, 25 g of sample was transferred into a blade bottle, to which 225 mL of dilution liquid was added. The mixture was blended and agitated to facilitate a homogenous state. For the liquid sample, the ten-fold diluted liquid sample, 1 mL was measured and transferred into the blade bottle. To which 9 mL of dilution agent was added to make the 100× deluted sample, and the dilution method was repeated to prepare a serial diluted 100×, 1000×, and 10000× samples.

Briefly, 1 mL of 10× diluted sample was placed into the *E. coli* and *E. coli*-like fast detection chip folder (3 M Biotech company), pressed with the pressing plate, and incubated at 37ºC for 48±2h. The control test was carried similarly using the same amount of dilution liquid. Duplicate experiments were performed and the number of *E. coli* like group bacilli was counted from the scope as defined and the results were expressed as cfu/g or cfu/mL.

Data processing and analysis

The data with Q & A were collected, complied into computer, and analyzed with EXCEL and SPSS software which were further treated with Wilcoxon statistics to compare the outcomes before and after the execution of GHP.

Results and Discussion

Over abuse of preservatives and the adulteration of illegal preservative

After receiving the GHP training under our PTSP from the year 2010 to 2014, the percent of underqualified cases [100×(positive case number/sampled number)] seemed to be almost unchanged (Table 1) at a glance, however the total cases of underqualified samples in fact have been greatly reduced to 4 only, contrasting with that before the GHP training (the year 2006-2008), during which a total illegal cases once substantially reached 233 cases (Table 2). In both periods, each showed a single case that used illegal dehydroacetic acid (DHAA) in the year of 2008 and 2011, respectively (Table 2). To our astonishment, in an alternate inspection of 21 samples from 11 factories before and after GHP training, the products that used sorbic acid as the preservative included soybean protein sheet and dried soybean curd, reaching 33.3% and 33.3% respectively. 33.3% of other soybean curd and Tofu products were added with benzoic acid as preservative, and the amount used were 0.12±0.07 g/kg and 0.13±0.09 g/kg, respectively (Table 3). Amazingly, some makers used illegal and prohibited preservatives like salicylic acid and DHAA before and even after the GHP training. Seven Tofu samples were found to contain salicylic acid as high as 0.19±0.02 g/kg (7 cases), and another Tofu samples (2 cases) were found adulterated with DHAA at a quantity as large as 0.16±0.02 g/kg. All other samples were found to contain only official preservatives like sorbic acid (2 cases), bezoic acid (4 cases), *p*-alkylhydroxybenzoate (1 case) at doses meeting the FDAT regulation (Table 2, Table 3).

With the increasing use of processed foods since the 19th century, there has been a great increase in the use of food additives of varying levels of safety. This has led to legislation in many countries regulating their use. FDAT has declared that the preservatives sorbic acid, benzoic acid and p-HBA can be officially used to preserve the dried soybean curd and soybean protein sheets, but not for Tofu and other related soybean products. The allowed dose was 2.0 g/kg for sorbic acid, 0.6 g/kg for benzoic acid and sodium benzoate, and 2.5 g/kg for all *p*-alkylhydroxybenzoates (Table 4). Although sulfur dioxide (0.3 g/kg) is allowed to be used in all soybean products including Tofu, no case had ever been found with sulfur dioxide (Table 4).

Table Captions

Table 1: Percent underqualified cases of Tofu and related products during the year from 2006 to 2014.

Number of cases over-abusing preservatives before receiving PTSP[a]				
year	**Total number sampled**	**Number examined**	**Number underqualified**	**Percent underqualified, %**
2006[a]	218	74	23	11
2007[a]	226	77	13	6
2008[a]	186	82	20	11
Total sum[a]	630	233	56	9
Number of cases over-abusing preservatives after receiving PTSP[b]				
2010[b]	32	2	0	0
2011[b]	27	14	4	15
2012[b]	32	11	2	6
2013[b]	22	8	2	9
2014[b]	59	12	6	10
Total sum[b]	172	47	14	8

[a]PTSP: Post-Market Training and Supervising Project
[b]This paper.

Table 2: The preservative specific case number found in Tofu and related products during the chronological year from 2006 to 2014.

Number of cases over-abusing preservatives before receiving PTSP[a]					
Year	**Sorbic acid**	**Benzoic acid**	**DHAA[c]**	**HPO[d]**	**subtotal**
2006[b]	32	27	11	4	74
2007[b]	31	36	7	3	77
2008[b]	60	10	9	3	82
Sum[b]	141	91	1	0	233
Number of cases abusing illegal preservatives after receiving PTSP[c]					
Year	**Sorbic acid**	**Benzoic acid**	**DHAA[d]**	**SalA[e]**	**subtotal**
2009[c]	-	-	-	-	-
2010[c]	0	0	0	3	3
2011[c]	0	2	1	0	3
2012[c]	2	1	1	4	8

2013[c]	0	1	0	1	2
2014[c]	0	0	0	0	0
Sum[c]	2	4	2	8	16

[a]PTSP: Post-Market Training and Supervising Project.
[b]Before PTSP and GHP.
[c]This apper.
[d]DHAA: dehydroacetic acid. [d]HPO: hydrogen peroxide
[e]SalA: salicylic acid

Table 3: Preservatives found in Tofu and related products in Taiwan before and after GHP guidance during the year 2010 to 2014.[a]

Preservative[b]	% prevalence[c], level added (g/kg)						
	Protein sheet	Dried soybean curd	Tofu A	Tofu B	Tofu C	Tofu D	Tofu E
HPO, before	0	0	0	0	0	0	0
after	0	0	0	0	0	0	0
SDO, before	0	0	0	0	0	0	0
after	0	0	0	0	0	0	0
FMDH, before	0	0	0	0	0	0	0
after	0	0	0	0	0	0	0
SorbA, before	33.3%; 0.71±0.08	33.3%; 0.96±0.07	0	0	0	0	0
after	0	0	0	0	0	0	0
BenzA, before	0	0	0	0	0	0	0
after	0	33.3%; 0.12±0.17	33.3%; 0.13±0.09	0	0	0	0
SalA, before	0	0	0	33.3%; 0.16±0.02	66.6%; 0.19±0.02	33.3%; 0.11±0.08	0
after	33.3%; 0.28±0.02	33.3%; 0.23±0.01	33.3%; 0.22±0.01	0	0	0	0
DHAA, before	0	0	0	0	0	33.3%; 0.16±0.02	0
after	0	0	0	0	0	0	0
PHBA, before	33.3%; 0.49±0.18	0	0	0	0	0	0
after	0	0	0	0	0	0	0

[a]GHP: good hygienic practice
[b]HPO: hydrogen peroxide. SDO: sulfur dioxide. FMDH: formaldehyde. BenzA: benzoic acid.
SalA: salicylic acid. DHAA: dehydroacetic acid. PHBA: *p*-hydroxybenzoic acid.
[c]% prevalence
= 100×(number of samples found with preservatives/number of samples)

Table 4: Official allowance of food preservatives

Preservative	Dried soybean curd	Soybean protein sheets	TOFU
formaldehyde	prohibited[a]	prohibited	prohibited
hydrogen peroxide	prohibited	prohibited	prohibited
salicylic acid	prohibited	prohibited	prohibited
dehydroacetic acid	prohibited	prohibited	prohibited
sorbic acid, g/kg	2.0	2.0	prohibited
potassium sorbate, g/kg	2.0	2.0	prohibited
sodium sorbate, g/kg	2.0	2.0	prohibited
calcium sorbate, g/kg	2.0	2.0	prohibited
benzoic acid, g/kg	0.6	0.6	prohibited
sodium benzoate, g/kg	0.6	0.6	prohibited
potassium benzoate, g/kg	0.6	0.6	prohibited
p-methylhydroxybenzoate, g/kg	2.5	2.5	prohibited
p-ethylhydroxybenzoate, g/kg	2.5	2.5	prohibited
p-propylhydroxybenzoate, g/kg	2.5	2.5	prohibited
p-butylhydroxybenzoate, g/kg	2.5	2.5	prohibited
p-isopropylhydroxybenzoate, g/kg	2.5	2.5	prohibited
p-isobutylhydroxybenzoate, g/kg	2.5	2.5	prohibited
Sulfur dioxide, g/kg	0.3	0.3	0.3

[a]prohibited and not listed.

Table 5: Reference standards of good hygienic practice for ranking the sanitation status of soybean products and manufacturing sites

Grade of sanitation	1	2	3	4	5	6	7
log cfu/g	< 1	1~ 2	2~ 3	3~ 4	4~ 5	5~ 6	> 6

Environmental and food sanitation problems

In the soybean product processing plants, the microbial contamination was severe, in particular, the Tofu manufacturing sites. By two GHP surveys, it reached a microbial population of log cfu/g 4- >6, with ranking 5-7 (Fig. 4, Table 5). Other sites for processing the dried Tofu curd, soybean protein sheets (SPS) and other products seemed to be less contaminated, gaining sanitation ranking at 3-7, 3-5, and 1, respectively. While the microbial contamination in the soybean products seemed to be less severe than that in the environment. Even so, the microbial contamination still reached a log cfu/g 1-4, 1, 1, and 1, respectively for Tofu, Dried Tofu Curd (DTC), SPS, and others, gaining the rank 1-4 for Tofu, 1 for DTC, SPS, and other products (Fig. 5, Table 5). Here, rank 1 means the the contamination reached log cfu/g < 1, rank 2 with a log cfu/g 1-2, rank 3 with log cfu/g 2-3, rank 4 with a log cfu/g 3-4, rank 5 with a log cfu/g 4-5, rang 6 with a log cfu/g 5-6, and rang 7 has the highest contamination index log cfu/g >6 (Table 5).

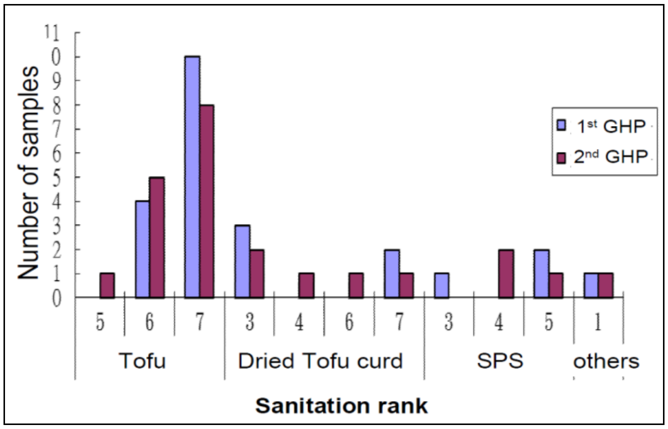

Fig 4: The sanitation ranking judged by the total bacterial count found in the first and the second GHP *in situ* soybean manufacturing sites.[a]
GHP: good hygienic practice. SPS: soybean protein sheet (rolls).

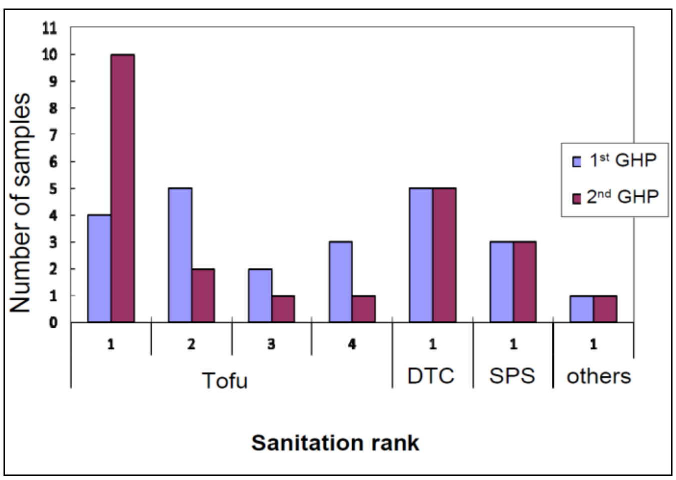

Fig 5: The *E. coli* count found in the soybean products for the first and the second guidance of GHP.

Tacket el al. investigated 50 *Yersinia enterocolitica* infections during an outbreak of illness due to contaminated Tofu (soybean curd) in Washington State between December 15, 1981 and February 22, 1982.
The most common clinical syndrome (36 patients) was gastrointestinal infection for which two patients underwent appendectomies and one a partial colectomy. Of the remaining 14 patients, six had extraintestinal infections, two had fever alone, and six were asymptomatic. Enteric infections more easily attacked the younger (median age three years), and extraintestinal infections had prevalence in the adults (median age 28 years) (Tacket *et al*., 1985) [46]. In the Tofu making sites, the transmission of *Y. enterocolitica* from nature to man and the potential of "natural" foods as vehicles for environmental pathogens has been confirmed by Tacket *et al*. (1982).
As contrast, *Staphylococcus aureus* bacteria are mostly transmitted via person-to-person contact and through food as a result of improper food handling and inadequate hygiene on the part of foodservice workers (About Food: *Staphylococcus aureus*). The foods involved in outbreaks of poisoning from *S. aureus* include meats and poultry, as well as other proteins such as eggs and tofu. Dairy products including milk and cream-filled pastries can also be contaminated. Finally, because the *S. aureus* toxin isn't killed by cooking, reheated foods of all kinds which have been handled by infected workers can also cause illness (About Food: *Staphylococcus aureus*).
Recently AnanChaipattana *et al*. reported the microbial contaminating status of Tofu products in Thai markets. Tofu samples (59 packaged tofu samples and 74 unpackaged Tofu samples) were collected and the contaminating bacilli were analyzed. The Gram-negative bacteria consisted of coliforms (67% in tested samples), *Pseudomonas* spp. (56%) and *Escherichia coli* (28%). The predominantly identified Gram-positive bacteria were *Enterococcus* spp. (77%), lactic acid bacteria (68%), *Bacillus cereus* (41%) and *Staphylococcus* spp. (26%) (AnanChaipattana *et al*., 2012) [1]. The contamination rates of *E. coli*, *Salmonella* spp. and *Enterococcus spp*. in unpackaged Tofu were significantly higher than those in packaged Tofu (p<0.05). Three kinds of Gram-negative pathogenic bacteria, *Cronobacter sakazakii*, *Salmonella spp*. and *Yersinia enterocolitica* (serotype 0:5), were found only in unpackaged Tofu samples. Among 54 *B. cereus* isolates, four isolates from three unpackaged Tofu samples and one packaged Tofu sample were found to produce diarrheal enterotoxin. The most common *Enterococcus spp*. isolates were *Enterococcus faecium* and *Enterococcus faecalis* (AnanChaipattana *et al*., 2012) [1]. Similar results have also been reported by Wang *et al*. (2009) [52]. By a survey of 26 samples from 8 Tofu manufacturing processes in Korea, the mean total viable counts in simply packed Tofu (the conventional Tofu) were found to be 5.3×10^6 cfu/g, and that of coli form was 2.9×10^3 cfu/g (Wang *et al*., 2009) [52]. As contrast, the mean total viable counts found in the packed Tofu were reported to be 6.1×10^4 cfu/g, and that of coli form counts were 2.0×10 cfu/g (Wang *et al*., 2009) [52], implicating the form of packing plays an important role in tofu sanitation. Wang *et al*. also pointed out that the process steps when soybean curd solution is being heated and being coagulated in reality exhibit the least total viable counts and *E. coli* counts, while the contamination was highest at steps of product forming, first cooling, the second cooling, and the simply packing (Wang *et al*., 2009). Further higher viability counts occurred at the beginning steps involving the steps of ‘soybean rinsing’, ‘soybean soaking’, ‘blended solution’, and the ‘filtrate’ (Wang *et al*., 2009) [52]. At the same time, Wang *et al*. also investigated the effect of storage time on the viable counts: within 3 days storage, 22.4% of the conventional Tofu samples reached a total viable counts of

22.4%, contrasting with 1.4% for the packed and sterilized Tofus (Wang *et al*., 2009) [52], indicating the post manufacturing packing form could play a very important role in quality evaluation of Tofu.

Toxicity of salicylic acid

Following oral administration, ASA (aspirin) is rapidly hydrolysed to salicylic acid, which is used in a wide range of cosmetic products. The plasma salicylate half-life following therapeutic doses is 2 to 4.5 h, but increases to 18 to 36 h in the case of an overdose (Done, 1960). In humans, intake of doses of >10 g ASA in adults or > 4 g in children can be fatal (Norwegian Medicines Agency [online]).

Oral doses of ASA of 100 mg/kg or higher induce salicylism (a toxic syndrome caused by excessive doses of ASA or any of the salicylates). Common symptoms are tinnitus, nausea and vomiting (Norsk Legemiddelhåndbok [online]).

In human the oral lethal dose for sodium salicylate is estimated between 20 and 30 g in adults (Goodman and Gilman, 1970) [15].

When the mated female rats were administered orally by gavage twice daily on gestation days 15 through 21 at dosage levels of 20, 80 and 200 mg/kg/day sodium salicylate at a volume of 20 ml/kg, administration of 200 mg/kg/day sodium salicylate and of 261 mg/kg/day acetylsalicylic acid (as positive control) induced maternal toxicity: agonal clinical signs and/or reduction of body weights and food consumption. These signs were generally associated with prolonged parturition and difficulty indelivery. A significant increase in mean gestation length was noted with acetylsalicylic acid. Corresponding adverse effects on offspring survival for the affected dams were noted. However, there was no evidence of systemic or maternal toxicity, and no adverse effects on offspring survival or growth in the mid-dose group (80 mg/kg/day) and in the low-dose group (20 mg/kg/day) (Procter & Gamble, 1994e *in*: SCCNFP, 2002) [40]. Under the experimental conditions adopted, the NOAEL (No-Observable-Adverse-Effect-Level) of sodium salicylate has been found to be 80 mg/kg/day when administered orally to mated rats corresponding approximately to 69 mg/kg/day of salicylic acid (SCCNFP, 2002) [40].

A total of seven cases were found committed to the use of salicylic acid, reaching a level of 0.19±0.02 g/kg in Tofus (Table 2, Table 3). Approximately 80% of salicylic acid is metabolised in the liver. Salicylic acid in conjugation with glycine, forms salicyluric acid and with glucuronic acid forms salicyl acyl and phenolic glucuronide. These metabolic pathways have only a limited capacity. Small amounts of salicylic acid are also hydroxylated to gentisic acid. With large salicylate doses the kinetics switch from first order to zero order (Levy *et al*., 1972) [30].

Toxicity of dehydroacetic acid

In human toxicity excerpts, DHAA causes impaired kidney function in humans. Large doses can cause vomiting, ataxia, convulsions (The Merck Index, 1976). At lower doses, no primary or allergic skin reactions. Human ingested 0.01 g/kg daily for 150 days without observable ill-effects (Gosselin *et al*., 1984) [16].

In non-human toxicity excerpts, a high dose levels monkeys showed anorexia, vomiting, weakness, stupor, ataxia, and convulsions (Gosselin *et al*., 1984) [16]. Loss of weight was perceived in rats fed with 0.3 g/kg of DHAA over 34 days but no ill-effects were found at 0.1g/kg for over 2 years (Spencer, 1982). We found the single case with 0.16±0.02 g/kg DHAA (Table 3) which might elicit ill effects to the consumers in the long run.

Literature elsewhere indicated that sodium dehydroacetate (120-300 mg/day for 3 days or 7 days i.p.) given to mice increased lipid droplets in centrilobular and midlobular hepatocytes. It produced little or no proliferation of smooth endoplasmic reticulum in hepatocytes and little increase in drug-metabolizing enzyme activity regarding sodium dehydroacetate (Kanai *et al*., 1981) [26]. The LD_{50} was 570 mg/kg of dehydroacetic acid for rat oral, or 1000 g/kg sodium DHAA hydrate for rat oral (Budavari, 1996), and dehydroacetic acid does not cause mutagenicity in any tester strain with or without metabolic activation (Inveresk, 1977) [23].

Toxicity of sorbic acid

Weak-acid preservatives, such as sorbic acid and acetic acid, are used in many low pH foods to prevent spoilage by fungi. The spoilage yeast *Zygosaccharomyces bailii* is notorious for its extreme resistance to preservatives and ability to grow in excess of legally-permitted concentrations of preservatives (Stratford *et al*., 2013) [45].

Sorbic acid and its salts have been subjected to an extensive battery of tests, including acute, short-term and chronic toxicity/carcinogenicity tests, two-generation reproduction and teratogenicity studies. These studies show that sorbic acid and sorbates have a very low level of mammalian toxicity, even in chronic studies at up to 10% of the diet, and are devoid of carcinogenic activity (Walker, 1990). Compared with the controls, there were slightly lower body weights in mice given 10% sorbic acid, increased relative kidney weights at the two higher levels and increased relative liver weights at all treatment levels (Hendy *et al*., 1976). Under some conditions, particularly at high concentrations or when combined with nitrites, potassium sorbate has shown genotoxic activity *in vitro* (Márcio *et al*., 2014). Although some research implies it has a long-term safety record (Tulamait *et al*., 2005), *in vitro* studies have shown that it is both genotoxic and mutagenic to human blood cells. Potassium sorbate is found to be toxic to human DNA in peripheral blood lymphocytes, and hence found that it negatively affects immunity (Sevcan *et al*., 2010).

Pure potassium sorbate is a skin, eye and respiratory irritant (Chem One, Ltd.). The maximum acceptable daily intake for human consumption is 25 mg/kg, or 1750 mg daily for an average adult (70 kg) (Márcio *et al*., 2014). Two cases were found to contain 0.17±0.08 g/kg and 0.96±0.07 g/kg, respectively during the GHP training (Table 2, Table 3). Sorbic acid is allowed to be used in dried soybean curd and soybean protein sheets at maximum of 2.0 g/kg, but it is prohibited for Tofu and other related products (Table 4).

Toxicity of benzoic acid

After uptake, benzoic acid and sodium benzoate are rapidly absorbed from the gastrointestinal tract and metabolized in the liver by conjugation with glycine, resulting in the formation of hippuric acid, which is rapidly excreted via the urine. Although its metabolism and excretion are rapid, an accumulation of benzoates or their metabolites is not to be expected, cases of urticaria, asthma, rhinitis or anaphylactic shock have been reported following oral exposure to benzoic acid and sodium benzoate (Maibach and Johnson, 1975; Anderson, 1996; Coverly *et al*., 1998).

Benzoic acid is a skin and eye irritant in rabbits. The acid and its sodium and potassium salts display a moderate to low acute oral toxicity in experimental animals (Bibra Toxicology Advice and Consulting 1989). Ingestion of benzoic acid has caused stomach pains, while the acid or its sodium salt taken orally may have provoked episodes of asthma and skin rash in people who suffer from these conditions. The acid had a moderate acute oral toxicity in man (Bibra Toxicology Advice and Consulting 1989). In experimental animals, repeated oral exposure to the acid or its sodium salt has caused effects on the central nervous system and damage to various organs including the spleen, lymph nodes, liver, kidneys, lungs, brain and gastro-intestinal tract (Safety Emporium; Bibra Toxicology Advice and Consulting 1989). It causes human skin irritation at 6 mg/kg, and the toxic dose low oral (TLDo) for human is 500 mg/kg (Safety Emporium). Foetal malformations were seen when pregnant rats were given repeated doses of sodium benzoate orally or by injection (Bibra Toxicology Advice and Consulting 1989). Long-term oral studies with the acid or its sodium salt gave no real evidence of carcinogenicity in rats or mice. Sodium benzoate caused chromosome damage in cultures of mammalian cells but did not affect the bone marrow chromosomes of rats *in vivo*, nor did it induce mutations in fruit flies or in Ames bacterial assays (Bibra Toxicology Advice and Consulting 1989). Examples of upper concentrations allowed in food are up to 0.1% benzoic acid (USA) and between 0.15% and 0.25% (other countries) (Chipley, 1983) [19]. The European commission limits for benzoic acid and sodium benzoate are 0.015-0.5% (EC, 1995). FDAT has issued the limit at 0.6 g/kg allowable for use in the dried soybean curd and soybean protein sheet (Table 4) and during the year 2010 to 2014, one case was found to violate the use of benzoic acid in Tofu, reaching a level of 0.13±0.09 g/kg (Table 3, Table 4).

It is worth noting that preservatives inhibit the great majority of yeast and mould species, but a few species are able to proliferate in preserved foods (Pitt and Hocking, 1997) [38]. These are the spoilage fungi, and their physiological properties largely define their spoilage behaviour. The most dangerous spoilage yeasts (Group 1) were characteristically preservative-resistant (Davenport, 1996) [11], osmotolerant, vitamin-requiring and highly fermentative, leading to excessive gas formation, bottle explosions, and occasional physical injury (Grinbaum *et al*., 1994) [19].

Zygosaccharomyces bailii has been reported to be highly resistant to sorbic, benzoic, acetic and propionic acids (Ingram, 1960; Malfeito-Ferreira *et al*., 1997; Neves *et al*., 1994; Pitt, 1974) and to sulphite (Goswell, 1986; Goto, 1980), especially in Taiwan whose climate is warm and moist.

Food safety is a complex issue that has an impact on all segments of society, from the general public to government, industry, and academia.

Toxicity of *p*-hydroxybenzoates

Hydroxy benzoate like 4-hydroxyethylbenzoate led to reactive oxygen species (ROS) formation in melanoma cells, its mechanisms of toxicity in SK-MEL-28 was o-quinone formation, intracellular GSH depletion, ROS formation and mitochondrial toxicity (Vad *et al.*, 2008) [50].

The joint FAO/WHO Expert Committee has indicated that all *p*-alkylhydroxybenzoates are safe to animals and humans and these substances have been evaluated for acceptable daily intake (Joint FAO/WHO, 1974).

The FADT regulation announced a level of 2.5 g/kg with respect to *p*-alkylhydroxybenzoic acid to use in the dried soybean curd and soybean protein sheets (Table 4).

Conclusion

Food safety is a complex issue that has an impact on all segments of society, from the general public to government, industry, and academia.

In this PTSP by executing GHP, we have found that the Tofu manufactories are apt to be polluted and contaminated with a huge population of microbes, which in turn may contaminate the product Tofu and related product. In order to extend the shelf life of Tofus, seven cases (samples) have been highly abused with the illegal preservatives like salicylic acid and dehydroacetic acid during the year 2110-2014. Alternatively, one case was found adulterated with *p*-alkylhydrxybenzoates. The absolute number of manufacturers that had been abusing the preservatives in fact was substantially reduced from 233 cases before GHP training (during 2006 to 2008) to merely 16 cases during GHP training (during the year 2010 to 2014). At the same time, the sanitation ranks of the environment *in situ* the Tofu manufacturing sites and the Tofu products were greatly improved, most of the product Tofus showed a sanitation rank of log cfu/g as low as 1-2, implicating the effectiveness of the GHP training.

Acknowledgments

不需要Acknowledgments 嗎?

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抱歉，此處不知如何修訂，期刊要求為[1]、[2,3]

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