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Histamine Production by *Lactobacillus rhamnosus*

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Abstract

Lactobacillus rhamnosus is commonly used in milks, yogurts, and probiotics for human. *L. rhamnosus* has been reported to produce histamine that caused a risk to human. In the study, *L. rhamnosus* produced histamine in milk, not in MRS. By TLC, HPLC analysis and chemical tests according to United State and British Pharmacopoeia, *L. rhamnosus* (10^7 CFU) produced histamine from 10.671±0.256 µg/ml to 12.639±0.517 µg/ml in glucose milk medium and 11.373±0.128 µg/mL to 13.622±0.169 µg/mL in free glucose milk medium after 12 day incubation and 30.206±0.417 µg/mL to 30.685±0.690 µg/mL in glucose milk medium and 30.099±1.295 µg/mL to 44.410±4.893 µg/mL in free glucose milk medium after 14 day incubation. The study suggested the milk products supplied with *L. rhamnosus*, like yogurts should be used as soon as preparation.

Keywords: *Lactobacillus rhamnosus*, histamine, detection, thin layer chromatography, high performance liquid chromatography

1. Introduction

Lactobacilli are gram-positive rods which have been studying to create a probiotic source for human because it is considered to have low pathogenicity (Salminen et al.2002). Most of work on *Lactobacillus rhamnosus* focused on benefits to against infectious diseases or improve human health (Robertson et al.2000, Gildberg et al. 1998, Austin et al.1995, Skjermo et al.1999, Gatesoupe FJ. 1994, Gildberg et al.1995, Nikoskelainen et al.2001). Many cases that people have symptom with allergy in some food such as yogurt, wine, .etc. The food which contains *Lactobacilli* strains which were reported to have the amount of biogenic amines content (Deepika et al.2014). Histamine has adverse reactions that affect the vascular and nervous systems (Boyer et al.1999, Shalaby et al. 1996, Silla et al. 1996).

Histamine is a biogenic amine that takes part in local immune responses, involve in regulation of intestine physiological functions in the gut and play a role as a neurotransmitter in the enteric nervous system (Marieb, 2001; Kim et al., 2009). Histamine is derived from the amino acid histidine via enzyme L-histidine decarboxylase as catalyst and vitamin B6 as the substrate (Niven et al. 1981, Epps et al. 1945, Riley et al.1968, Rosenthaler et al.1965). This reaction is described as decarboxylation which removes a carboxyl group and releases carbon dioxide (Niven et al. 1981, Epps et al. 1945, Riley et al.1968, Rosenthaler et al.1965). Consequently, food like yogurt that is rich of L-histidine and vitamin B6 will be a histamine source.

There are many histamine producing bacteria which were isolated and studied, such as *Morganella morganii, Photobacterium phosphoreum, Raoultella planticola, Serratia liquefaciens, Proteus mirabilis* (Tsai et al. 2005). However, the ability to produce histamine in bacteria depends on environment such as: animal, fish, etc (Visciano et al.2012). Even there are some strains such as *Bacillus subtilis*, a strain is believed to be a perfect probiotic source, also produce histamine (Yue Hu et al. 2014). Histidine decarboxylase enzyme activities is vigorous in high temperatures and kept its activity to slightly below 5°C. Therefore, to conclude whether the products containing histamine needs more times for the fermentation to detect concentration histamine threshold. (Razavi, 1994).

Comparing to agar medium which was developed by Niven et al., (1981), these above media were used to culture the target bacteria. However, the histidine with known percentage and bromocresol purple as an indicator to detect the histamine producing bacteria are not changed. For the previous study, 36- 72hr at 28°C incubation period was carried out (Niven et al. 1981).

Lactobacillus rhamnosus is used in many probiotic products. However, the detection of histamine in these products was not mentioned (Yue Hu et al. 2014). Therefore, this study focused on conditions relating to histamine production and detection in *Lactobacillus rhamnosus.*

2. Material and Methods

2.1 Strains and media

Lactobacillus rhamnosus ATCC 11533 was used in the study. The cultures were inoculated on MRS agar with 1.0% histidine and stored at 20°C (Wei et al. 1989). Single colonies were sub-cultured continuously in order to determine histamine.

2.2 Optimizing condition for histamine production

The *Lactobacilli* MRS and milk culture media were added 1.0% histidine, 0.006% bromocresol, 0.003% vitamin B_6 depended on bacterial genetic characteristics as well as the ways to synthesize histamine. The pH was adjusted to 6.5± 0.2 in the practical culture medium which then was sterilized at 121°C for 15 minutes. The amount of 10^7 CFU was inoculated into culture medium broth at room temperature. Consequently, 10mL of culture broth were taken for analysis at sampling time of 10, 12, 13, 14 days.

2.3 Histamine extraction

Histamine was released in the culture medium, hence 10mL samples were centrifuged to isolate pellet including extracellular protein, cells, etc. Based on the solubility of histamine, supernatant were filtered through 0.45µm filter papers and shook up 5 mL hot chloroform (2:1) treated with 60^0C. The process was repeated in 3 times. Then chloroform layers were gathered and continuously shook up 5 mL ethanol (1:1). The process were repeated in 3 times. Then the solution were allowed to stand for ethanol evaporation completely.

2.4 Histamine detection

2.4.1 Morphological detection

For histamine production on agar media, *L. rhamnosus* was cultured on MRS and milk agar added 1.0% histidine, 0.006% bromocresol, 0.003% vitamin B_6. Colony morphology was checked on agar and under microscope.

2.4.2 Detection histamine by color forming characteristics

According to the United States Pharmacopeia 23**,** the final solution, which was believed containing histamine, was added with mixture of water and 1 N sodium hydroxide with rate 7:3. Then the solution was treated with a mixture of 50 mg of sulfanilic acid, 10mL of water, 2 drops of 2M hydrochloric acid, and 2 drops of 2M sodium nitrite solution. A deep red color was produced.

2.4.3 Detection histamine by precipitation characteristics

According to the British Pharmacopoeia 1993, the final solution was acidified with 2 M nitric acid and 0.4 mL of silver nitrate solution. Then the solution was shook for five minutes and allowed to stand. A curdy, white precipitate was produced.

2.4.4 Thin layer chromatography (TLC)

TLC method was used to detect histamine which had two phases: stationary and mobile phases. The stationary phase was silica plate gel 60 (Muangthai et al.2014). Based on the reference materials, the solubility and polarity of histamine, there were several mobile phases for histamine detection. However, only two systems were developed due to the limited condition and duration in thesis research. Therefore, chloroform, methanol and ammonium were considered for experiment. The ratio of solvents were described in the below table.

Table 1: The ratio of the solvents for histamine detection.

Mobile phase 1			*Mobile phase 2*	
$CHCl_3$	CH_4O	NH_3	CH_4O	NH_3
12	7	1	19	1
12	8	1	18	2
12	9	1	17	3
12	9	2	16	4
12	9.2	2.2	15	5

The spots on the chromatogram were detected by spraying with ninhydrin reagents. The dark purple spot and retention factor was considered histamine. (Bjornsdottir et al.2009).

2.4.5 Histamine quantitation

The histamine which was produced by *Lactobacillus rhamnosus* after fermentation were analyzed using the HPLC method. The detection and quantitation by high performance liquid chromatography, using mobile phase water: acetonitrile (92:8) with flow rate 0.8ml per minute (Jahedinia M. et al 2014). The samples were determined with ultraviolet detector at 210nm and adopted a C18 column (Gemini 110A, 250×4.6, 5µm) for component separation, the analysis time for each sample was twenty minutes. (Jahedinia M. et al 2014). The results were recorded by Shimadzu LC solution software (Tokyo, Japan).

3. Results

3.1 Morphological studies

On modified agar, *L. rhamnosus* colonies produced yellow pigments, not purple color as reported in many previous studies. It was meant that *L. rhamnosus* produce organic acids in the media, leading low pH, therefore, although bromocresol was used as indicator in the agar media to observe the purple color change due to the pH increase in case *L. rhamnosus* produce histamine in bacteria. Therefore, bromocresol modified in agar for histamine detection reported previously that can not be used in histamine detection in *L. rhamnosus* or *Lactobacilli* genera. To study on histamine production in *Lactobacilli*, many tests should be done, like chemical tests, thin layer chromatography, high performance liquid chromatography analysis.

3.2 Chemical detection

For the United State Pharmacopoeia 23, the deep red color was detected in histamine standard and MRS samples. The extracts prepared from histidine modified MRS or milk were used as the negative control samples.

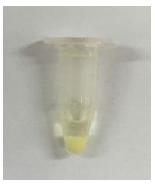
In modified milk condition, the samples had the same color with the histamine standard. Moreover, the negative control samples only had the yellow color that was different from others. The result for modified MRS medium failed to

show the positive color. The result for modified milk medium gave the positive color. It was meant that histamine was produced from the modified milk medium in both glucose and non-glucose conditions.

For the British Pharmacopoeia 1993, a curdy, white- purple precipitant was produced which gave the positive result in histamine standard. For the modified MRS sample, there was no white- purple precipitant that pointed modified MRS was not a suitable for histamine – producing *L. rhamnosus*.

The samples prepared milk extraction formed the white precipitants that gave the positive results of histamine. There was no observation for precipitation for negative control was considered to be logically. The summaries of histamine reaction tests in modified milk media that were suspected for histamine production were recorded in figure 1.

Figure 1: Histamine reaction based on the color forming and precipitation characteristics in milk.

	Standard	Samples	Negative
Color forming			
Precipitation			

3.3 Thin- layer chromatography analysis

Two developing system for mobile phase in thin layer chromatography was optimized by examining variable ratio. The best result determined the optimized system for 2% glucose- milk medium was CHCl3:CH4O:NH3 with the ratio 12:9:2 v/v respectively. And the optimizing mobile phase for non- glucose milk medium was 12:9.2:2.2 v/v

From figure 2, by TLC analysis, milk containing 2% glucose and non- glucose showed spots that are similar to standard histamine (Rf=0.5) (Figure 2).

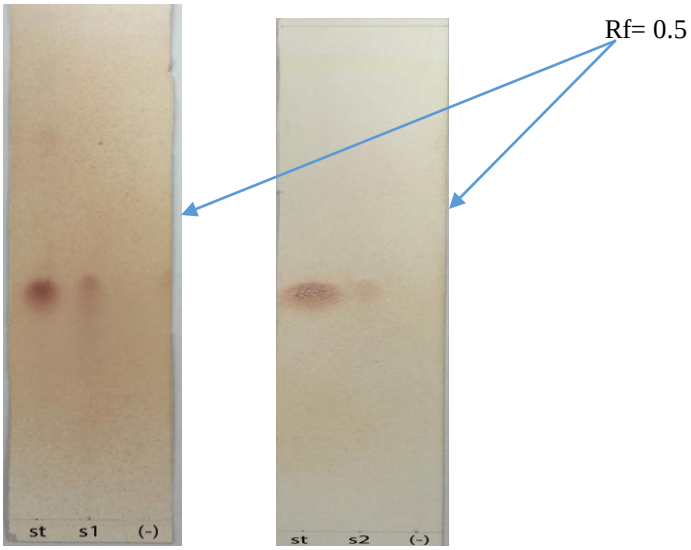


Figure 2: TLC of samples prepared from modified milk. (St) represents histamine standard spot; (s1), (s2) represents sample spot and (-) represents negative control.

The Thin layer chromatography result was summarized in the table 2.

Table 2: Histamine detection by thin layer chromatography in modified MRS medium and modified milk medium

	MRS	*Milk with glucose*	*Milk without glucose*
Day 10	(-)	(+)	(+)
Day 11	(-)	(+)	(+)
Day 12	(-)	(+)	(+)
Day 13	(-)	(+)	(+)

As showing in table 2 and figure 2, *L. rhamnosus* could not produce histamine in modified MRS while histamine was produced in modified milk. Probably, milk is rich of nutrients and some other substrates that make *L. rhamnosus* produce histamine. With this study, it is careful to use long stored milk supplied with *L. rhamnosus*.

3.4 Histamine quantification

The extracted solutions were introduced to high performance liquid chromatography (HPLC) for histamine detection and quantification. The data obtained from HPLC results was analyzed by comparison with the histamine standard. According to HPLC chromatogram, the retention time records were summarized in Figure 3 and Table 3.

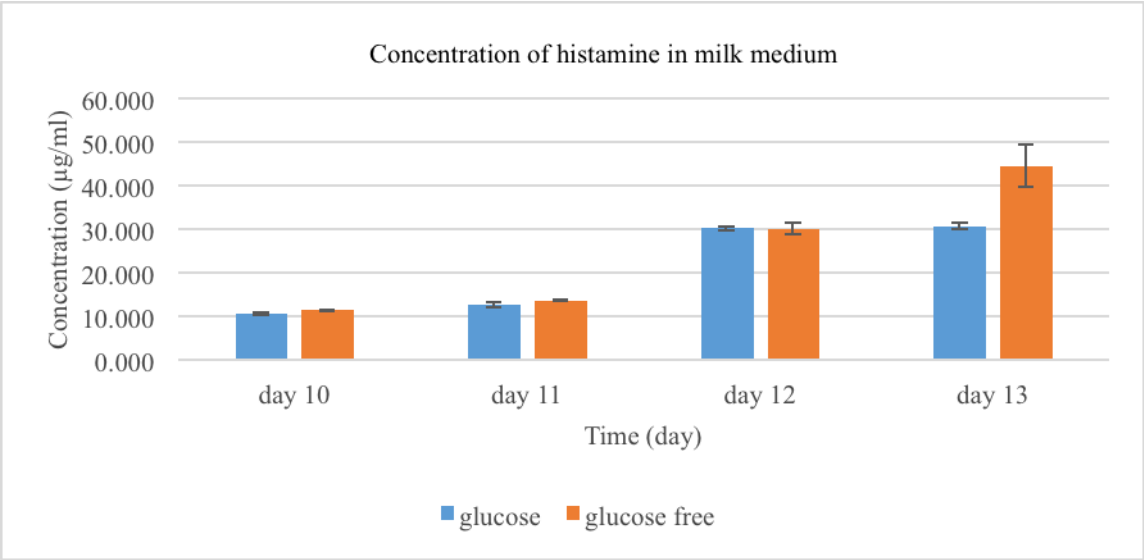


Figure 3: The graph of histamine concentration in milk medium with and without glucose which were measure in 10, 11, 12, 13 days of incubation

Table 3: The histamine concentrations obtained by the retention times

Time	**Concentration (µg/ml)**	
	Glucose	*Non-glucose*
Day 10	10.671±0.256	11.373±0.128
Day 11	12.637±0.517	13.622±0.169
Day 12	30.206±0.417	30.099±1.295
Day 13	30.685±0.690	44.410±4.893

With the known concentration of histamine loaded (324 µg/mL), data showed the concentration of histamine in the samples increased from 10.671±0.256 µg/mL to 12.639±0.517 µg/mL in glucose milk medium and 11.373±0.128 µg/mL to 13.622±0.169 µg/mL in free glucose milk medium. This finding therefore proved that there was the histamine production in milk medium in all treatments (glucose and glucose free medium) and the histamine continuously produced. In 12 and 13 days, although the retention time was slightly changed in standard peak, the followed samples were altered that fixed to the new standard peak. With the same concentration of histamine loaded, the histamine concentration in the samples were concluded to increase. Moreover, based on the showed data, the day 13 and 14 slightly increased from 30.206±0.417 µg/mL to 30.685±0.690 µg/mL in glucose milk medium and 30.099±1.295 µg/mL to 44.410±4.893 µg/mL in free glucose milk medium. The difference between two treatments was statistically analyzed. The obtained results revealed that there was no significant difference between two groups: glucose and non- glucose medium using multiple comparisons of means by Post hoc test (SPSS 22, SPSS Inc., Chicago, USA). The result of statistical analysis showed that there was a significant difference in the histamine production during 4 practical days ($p < 0.05$). Moreover, the concentration of histamine in day 10 and 11 were considered to be no significant change based on homogeneous subsets (SPSS 22, SPSS Inc., Chicago, USA).

According to the figure, the histamine production in glucose milk medium slightly change in day 12 and 13 while the histamine production in non- glucose milk medium change critically. This means without glucose, *Lactobacillus rhamnosus* ATCC 11553 had the potential to produce histamine continuously. It was meant that *L. rhamnosus* had some differentiation in glucose that prevent histamine production.

In general, this study was preliminary detected that histamine production existed in *Lactobacillus rhamnosus* ATCC 11553. The amount of histamine production were not high: 44.410±4.893 µg/mL for the highest within 4 experimental days which was not exceed 200 µg/mL. This data assessment were obtained from Thai national food consumption survey (ACFS. 2007), histamine in fish sauces report (Thai Department of Fishery), a histamine outbreak report (Bureau of Epidemiology, Ministry of Public Health, Thailand. 2007) and many published scientific literature and reports produced by various organizations. (Van Geldern et al. 1992). This can be concluded that although *Lactobacillus rhamnosus* produced small amount of histamine, there was not significantly affect to human health by allergy in probiotic products, food, milk or vegetables with the considered glucose component.

4. Conclusion

Lactobacillus rhamnosus could produce histamine in a rich nutrient medium, like milk, yogurt. Therefore, it is important to use these products as soon as possible. Also, a policy for storage these products should be noticed. For pharmaceutical field, *L. rhamnosus* formulation should be established in order to make sure the safety for human.

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