CHAPTER 2

MATERIALS AND METHODS

Materials

1. Stock culture of Lactobacillus casei, ATCC (American Type Culture Collection) No. 7469, obtained from American Type Culture Collection, 2112 M. Street, Washington, D.C.

2. Microbiological Assay Inoculum Broth Dehydrated or Maintenance Medium. (Fisher Scientific, Fair Lawn, N.J., U.S.A.)

3. Folic Acid Assay PGA Broth.

3.1 Microbiological Culture Medium. (Baltimore Biological Laboratory (BBL), Cockeysvilles, Maryland, U.S.A.)

3.2 Folic Acid Casei Medium. (Difco Laboratories, Detroit, Michigan, U.S.A.)

4. Chemicals.

4.1 Ascorbic acid, A.R.

4.2 Chicken Pancreas. (Difco Laboratories, Detroit, Michigan, U.S.A.)

4.3 Concentrated sulfuric acid, Lab. grade.

4.4 Disodium hydrogen phosphate (Na_2HPO_4), A.R.

4.5 Ethanol.

4.6 Folic acid. (Koch - Light Laboratories Ltd., Colnbrook Buckinghamshire, England.)

4.7 Lysol.

4.8 Potassium dichromate, Lab. grade.

4.9 Sodium dihydrogen phosphate dihydrates ($NaH_2PO_4 \cdot 2 H_2O$), A.R.

4.10 Sodium hydroxide, A.R.

4.11 Tween 80. (Atlas Powder Co., Welmington, Del.)

4.12 Gardinol Type Detergents. (Teepol.)

5. Instruments.

5.1 Analytical balance (Harvard Trip Balance, Ohaus Scale Corp. Union, N.J.)

5.2 Autoclave. (Prestige)

5.3 Automatic syringe.

5.4 Centrifuge. (International Portable Refrigerated Centrifuge, Model PR - 2)

5.5 Deep freezer. (Low Temperature Chest Model CA-280A, Chinishi Netsugaku, Co. Ltd. Japan.)

5.6 Electric Balance. (E. Mettler, Type H 16)

5.7 Hot air oven. (Thelco, Model 17)

5.8 Incubator. (Thelco, Model 6M)

5.9 Micropipetting system (50 and 100 microlitres) with disposable plastic tips. (Oxford Sampler, Model Q, Oxford Laboratories, Inc.)

5.10 Mixer. (Vortex - Genie Scientific Industries Supplies, Massachusetts.)

5.11 Nephelometer. (Nepho-Colorimeter, Model 9, Coleman Instruments Corporation.)

5.12 pH meter. (Beckman)

5.13 Refrigerator.

5.14 Water bath. (Chicago Surgical and Electrical Co., Division of Labline, Inc., Illinois)

Methods.

All glasswares used in the present experiment must be free from folate by boiling in Gardinol Type Detergents solution (1:200) for at least 30 minutes. They were then soaked overnight in the cleansing solution and rinsed well with tap water and distilled water.

Deionized - distilled water was used for preparation of all media and buffers. It was free from folate and the heavy metal which might have the undesirable ability to accelerate oxidation of the ascorbic acid in the buffer.

1. Preparation of Micro Inoculum Broth. (Maintenance Medium)

Five hundred millilitres of maintenance medium was generally prepared by dissolving 18.5 g of Maintenance Medium in deionized-distilled water up to 500 ml. Then the solution was dispensed into 50 screw-capped tubes. The tubes, with screw caps loosely affixed, were autoclaved at 118°C for 30 minutes. They were then left to cool at room temperature. The screw caps were tightened and the tubes were incubated at 37°C overnight. The following morning they were examined for clarity, indicating the absence of bacterial contamination and they were then stored in the refrigerator (4°C).

2. Maintenance of Stock Culture of Lactobacillus casei.

Lactobacillus casei ATCC (American Type Culture Collection) No. 7469 was kept in the maintenance media and stored in the refrigerator (4°C). The organism must be subcultured every 2 weeks by aseptically adding one drop of the stored liquid culture to fresh maintenance medium and incubated at 37°C for 18 hours. Then the culture was stored in the refrigerator (4°C).

Occasionally, the culture was checked for purity by plating on Blood Agar Medium and stored at 4°C.

3. Preparation of Inoculum. (Herbert, 1966)

In the afternoon before the assayed day, 1 drop of the stored culture was added to 10 ml of maintenance medium and incubated at 37°C for 18 hours. Then 0.5 ml of this fresh 18-hour culture was added to 10 ml of maintenance medium and incubated at 37°C for 6 hours. The inoculum for the assay, called "L.C." (Lactobacillus casei), was prepared by adding 0.05 ml of this six-hour-culture to 18 ml of the single strength basal medium. One drop of the inoculum was added to each assay bottle.

4. Preparation of Ascorbic Acid Phosphate Buffer.

This buffer must be freshly prepared by dissolving 150 mg ascorbic acid in 100 ml of phosphate buffer pH 6.1.

5. Preparation of Phosphate Buffer pH 6.1 (Herbert, 1966)

5.1 Solution A (acid 0.2 M) was prepared by dissolving 31.2 g of sodium dihydrogen phosphate dihydrates ($NaH_2PO_4.2H_2O$) in the deionized-distilled water and made up to 1 litre.

5.2 Solution B (base 0.2 M) was prepared by dissolving 28.4 g of Disodium hydrogen phosphate (Na_2HPO_4) in the deionized-distilled water and made up to 1 litre.

Then 212.5 ml of solution A was mixed with 37.5 ml of solution B and made up to 1 litre with the deionized-distilled water. The pH of the solution should be 6.1. The solution was stored at the room temperature.

6. Preparation of Conjugase. (Hurdle, 1968)

Three hundred milligrams of "Difco" desiccated chicken pancreas was dissolved in 100 ml of deionized-distilled water. The solution was centrifuged at 2,500 rpm for 10 minutes and the precipitate was discarded.

7. Preparation of Cleansing Solution.

Ten grams of Potassium dichromate was dissolved in 75 ml of distilled water and 25 ml of concentrated sulfuric acid was gradually added.

8. Preparation of Samples.

8.1 Human milk.

Milk samples from lactating mothers, second to twelfth day after delivery, were obtained from the Obstetrics ward, Rajvithi Hospital. Samples were collected in the morning by manual expression. The milk was frozen within one hour of collection and was thawed before assayed by the "General Method for Preparation of Sample".

8.2 Cow's milk.

8.2.1 Fresh cow's milk samples were obtained from dairy farms at Kasetsart University and Suan-Chitrlada Palace. The sample was frozen within one hour of collection and was thawed before assay. Then the thawed-milk was divided into 3 portions.

The first portion was proceeded as in the "General Method for Preparation of Sample".

The second portion was pasteurized at 62.8°C for 30 minutes (Lampert, 1975) and proceeded as in the "General Method for Preparation of Sample".

The third portion was sterilized at 15 lbs/in^2 pressure (121°C) for 5 minutes and proceeded as in the "General Method for Preparation of Sample".

8.2.2 Pasteurized milk and sterilized milk samples were purchased from the market. The sample was frozen and thawed before proceeded as in the "General Method for Preparation of Sample".

8.3 Other Cow's Milk Preparations.

8.3.1 Sweetened condensed milk and unsweetened condensed milk samples were purchased from the market and proceeded as in the "General Method for Preparation of Sample".

8.3.2 Powdered milk samples were purchased from the market. Five grams of each sample was dissolved in the warmed-deionized-distilled water (about 50°C) up to 30 ml suspension. Then proceeded as in the "General Method for Preparation of Sample".

8.3.3 Butter and cheese samples were purchased from the market and proceeded as in the "General Method for Preparation of Sample".

"GENERAL METHOD FOR PREPARATION OF SAMPLE"

Two millilitres of milk sample, milk suspension or two grams of sweetened condensed milk, butter or cheese was diluted to 20 ml with the ascorbic acid phosphate buffer and warmed if necessary. Then the solution was autoclaved at 15 lbs/in^2 pressure (121 °C) for 5 minutes. The solution was centrifuged at 2,500 rpm for 10 minutes and the precipitate was discarded. Five millilitres of the supernatant was numbered "U" and was frozen until assay. Another 9 ml of the supernatant was added to 1 ml conjugase and 0.5 ml of Toluene was added to cover the surface of the solution. It was incubated at 37°C for 16 hours. The solution was then boiled in water bath for 10 minutes and centrifuged at 2,500 rpm for 10 minutes.

The supernatant was frozen until assay. This portion was labelled "C" (conjugates) in the assay procedure.

9. Preparation of Folic Acid Assay PGA Broth. (Double strength)

The ready-made assay medium was stored in the refrigerator ($4^{\circ}C$) to avoid deterioration. The medium 9.4 g was dissolved in 100 ml deionized-distilled water and 1 drop of Tween 80 was added to the solution to lower the surface tension. The solution was boiled for 1 minute and cooled to room temperature. Then 50 mg of ascorbic acid was dissolved in the solution and filtered through a filter paper. Three millilitres of this medium was transfered to an assay bottle by the automatic syringe.

10. Preparation of Standard Folic Acid Stock Solution, 1.0×10^{-5} g/ml.

One hundred milligrams of standard folic acid powder was weighed and dissolved in 20 ml of deionized-distilled water. 0.1 N Sodium hydroxide solution was slowly added until the solution became clear yellow. The solution was made up to 100 ml with deionized - distilled water in the volumetric flask. The concentration of this solution was 1.0×10^{-3} g/ml. Then 1 ml of the solution was transfered into 100 ml volumetric flask and made up to 100 ml with 20 % ethyl alcohol. The concentration of the final solution was 1.0×10^{-5} g/ml. The solution was divided into 60 small tubes and stored at $-20^{\circ}C$.

11. Preparation of Standard Folic Acid Solutions, 1.0×10^{-9} g/ml and 1.0×10^{-10} g/ml

(These solutions must be freshly prepared before use.)

The standard folic acid stock solution (1.0×10^{-5} g/ml) was taken from the freezer, thawed and 1 ml of the solution was pipetted into 100 ml volumetric flask. The solution was made up to 100 ml with deionized-distilled water. The concentration of this solution was 1.0×10^{-7} g/ml

To prepare 1.0×10^{-9} g/ml folic acid solution, 1 ml of the 1.0×10^{-7} g/ml folic acid solution was diluted to 100 ml with deionized-distilled water in the volumetric flask. Then 10 ml of the 1.0×10^{-9} g/ml folic acid solution was diluted to 100 ml with deionized-distilled water in the volumetric flask. The concentration of the final folic acid solution was 1.0×10^{-10} g/ml.

12. Method of Assay Folic Acid.

12.1 The bottles for assay standard folic acid were numbered and prepared by adding deionized-distilled water, 1.0×10^{-9} g/ml standard folic acid solution and 1.0×10^{-10} g/ml standard folic acid solution (see 2.11) to each bottle as shown in Table 1.

12.2 The unknown assay bottles were numbered and prepared by adding deionized-distilled water to each bottle as shown in Table 2.

TABLE 1

VOLUME FOR ADJUSTMENT OF STANDARD ASSAY BOTTLES.

Bottle No.	Number of bottle	Final conc^{n} pg/ml	Std. folic acid sol^{n} (ml)		Deionized-distilled water (ml)
			$1.0x10^{-10}$ g/ml	$1.0x10^{-9}$ g/ml	
Control	1	0	-	-	3.0
0	3	0	-	-	3.0
1	3	5	0.3	-	2.7
2	3	10	0.6	-	2.4
3	3	20	1.2	-	1.8
4	3	40	2.4	-	0.6
5	3	70	0.2	0.4	2.4
6	3	100	-	0.6	2.4
7	3	150	-	0.9	2.1
8	3	200	-	1.2	1.8
9	3	300	-	1.8	1.2

TABLE 2

VOLUME FOR ADJUSTMENT OF UNKNOWN ASSAY BOTTLES.

Sample	Bottle numbered (duplicate)	Deionized-distilled water (ml)	Prepared sample without conjugase (ml)	Prepared sample with conjugase (ml)
unknown No.1	U_1	2.95	0.05	-
	$\bar{U}_1$	2.90	0.10	-
	C_1	2.95	-	0.05
	$\bar{C}_1$	2.90	-	0.10
unknown No.2	U_2	2.95	0.05	-
	$\bar{U}_2$	2.90	0.10	-
	C_2	2.95	-	0.05
	$\bar{C}_2$	2.90	-	0.10
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.				
.				
etc.				

Note : The prepared samples were added after sterilization.

12.3 With an automatic syringe, 3 ml of double strength assay medium (see 2.9) was added to each assay bottle.

12.4 Another 25 ml tube, 9 ml of deionized-distilled water and 9 ml of the double strength assay medium were added. This tube was marked "L.C."

12.5 All of the assay bottles and L.C. with screw caps loosely affixed were autoclaved at 15 lbs/in^2 pressure (121°C) for 5 minutes, cooled to room temperature and the screw caps were tightened.

12.6 The prepared samples were taken from freezer, thawed at room temperature and were aseptically added to each assay bottle by micropipette.

12.7 To prepare inoculum, 0.05 ml of six-hour culture (see 2.3) was added to the "L.C." bottle. One drop of this inoculum was inoculated to the assay bottles except the "control" bottle. This must be done with aseptic technique. Then the assay bottles were incubated at 37°C for 40-48 hours.

12.8 The density (absorbance) of the growth micro-organisms in each bottle was measured with Nepho-Colorimeter using a red filter (wave length = 640-700 nm) (Herbert, 1961) to reduce error due to the color of the medium.

12.9 The growth densities (absorbance) of each standard concentration were averaged and plotted on a graph paper to make the standard growth curve. The concentration of unknown were calculated from this curve.

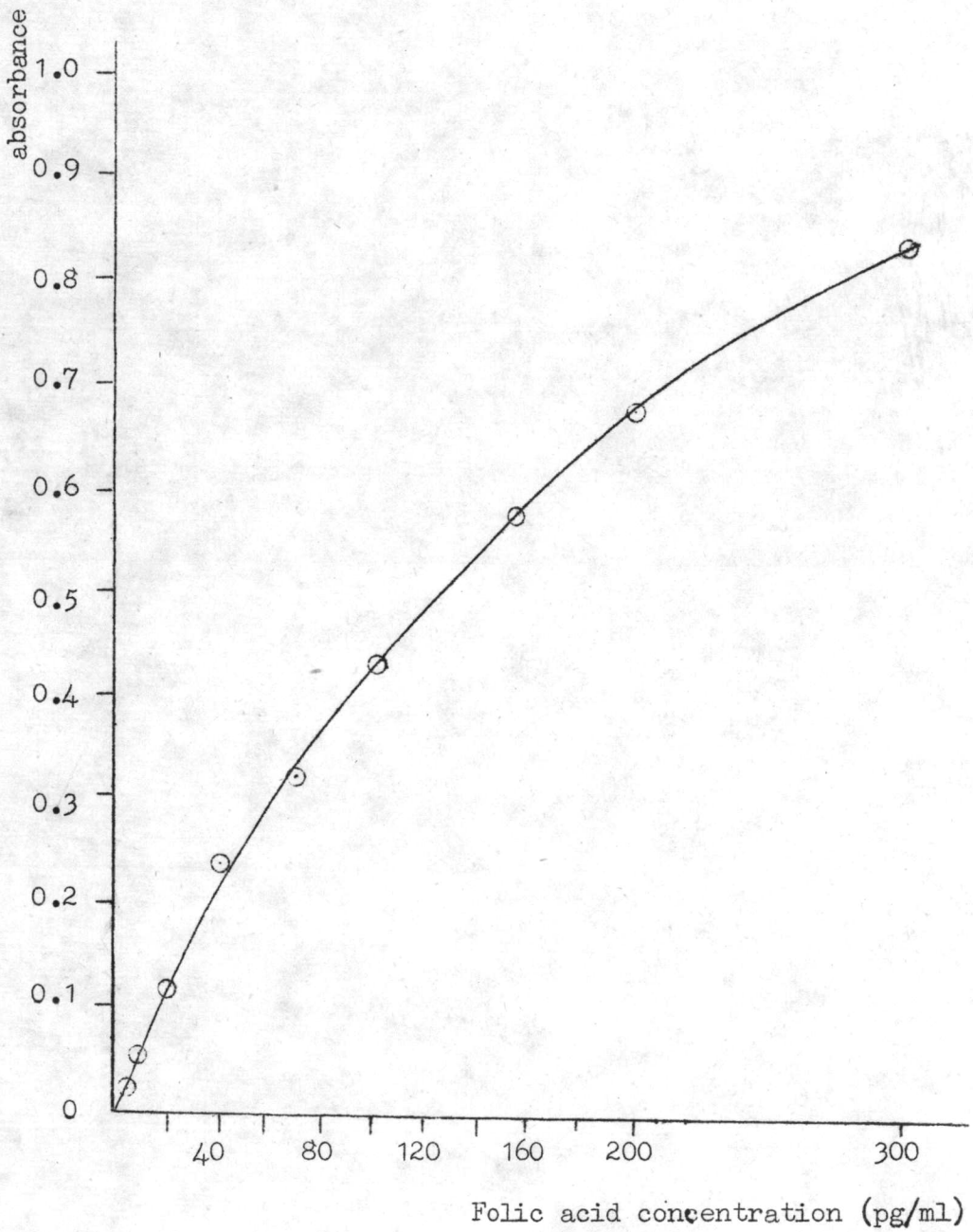

Figure 2

Standard curve of folic acid concentration vs. growth density (absorbance)