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The Isolation and Evaluation of Potential Probiotic Strains from Breast Milk

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Abstract: *In this newspaper, we want to isolate lactic strains have potential probiotic from breast milk. As a result, 22 lactic strains were isolated from 7 samples (breast milk) and chose 8 strains had potential probiotic. These 8 lactic strains survived in hard conditions such as acid stomach, bile salt and resisted to bacteria with diameter clear zone 6 mm-15 mm. Besides, they had high lactate and adhesive activity. To obtain lactic strains had various probiotic properties, 4 lactic strains were selected and determined by 16S rDNA, they included Lactobacillus paracasei HV3.2; Lactobacillus paracasei HV4.1; Lactobacillus acidophilus HV4.2 and Lactobacillus rhamnosus M3.2.*

Keywords: *lactic strains, probiotic properties, lactate activity, breast milk*

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I. Introduction

The most recent definition of "probiotic" was formulated by FAO/WHO (Food and Agriculture Organization/ World Health Organization) and refers to viable, non-pathogenic microorganism that when ingested in adequate amounts, are able to reach and colonize the Gastro-Intestinal tract and to confer health benefits to the host [1]. Besides, probiotic have precious properties such as resisting bacteria, decreasing cholesterol, preventing cancer and immune modulation.

Probiotic bacteria strains were isolated from vegetable fermented food, fermented milk; besides, breast milk is also a potential probiotic source. Breast milk contains 700 lactic strains most are *Streptococcus, Lactococucs*, *Leuconostoc* and *Staphylococcus.* They contribute to decrease harmful microorganisms, to promote growth and stability of beneficial bacteria. Therefore; probiotic bacteria strains from breast milk support babies have gut health [2].

Probiotic bacteria strains from breast milk are potential probiotic, but they were not studied widely in the world and Vietnam. Therefore, our research in this newspaper was performed to isolate and evaluate potential probiotic strains from breast milk.

II. Materials And Methods

2.1. Materials

- ✓ 4 samples (breast milk) considered HV1, HV2, HV3, HV4 provided by Hung Vuong hospital. HV1 provided by operated born. HV2, HV3, HV4 provided by normal born.
- ✓ M1, M2, M3 sample are provided by mothers raising babies in 1 month, 2 months and 3 months.

2.2. Research methods

2.2.1. Preparation of samples and activation LAB strains in breast milk

Samples (breast milk) are collected from mothers, they are contained in sterile polyethylene bags and stored in 4^0C-6^0C. Then, these samples are carried to the laboratory.

Samples (breast milk) add to sterile MRS broth (1 ml/4 ml) to activate probiotic strains in breast milk during 2 hours, under anaerobic condition at 37^0C [4].

2.2.2. Isolation of LAB

After activating samples, 1 ml samples was added to sterile physiological water (0.9% NaCl) and was further serially diluted. Then, 0.1 ml liquid of the samples suitable dilutions were plated onto MRS agar. The plates were incubated in 37^0C during 48 hours. After two days, the resulting isolates were randomly selected from the medium surface and were streaked on fresh MRS to be purified [3].

2.2.3. Phenotypic and genotypic identifications

All of the isolated strains, the colony morphology on MRS solid medium was determined visually. Gram staining was performed to determine the cell morphology and Gram stain reaction of the isolates [3].

2.2.4. Acid and Bile Salt Tolerance Assay

Acid tolerance

Preparing Simulated Gastric Juice (SGJ) at pH 2.5 in test tube and sterilized in 121^0C. Samples were added to SGJ at 10% the volume of SGJ and incubated in 0,1,2, 3 hours. Samples were diluted to 10^6 and spread on the plates to count colonies, then calculated colony forming units (CFU/ml) lactic strains survive at pH 2.5 and colony forming units (CFU/ml) lactic strains in control sample [5].
The percent of survival= colony forming units survive/colony forming units in sample control*100
Bile Salt Tolerance
Lactic strains are evaluated bile salt tolerance in above experiment. Lactic strains were cultured in SGJ medium added 0.3% bile salt and incubated in 37^0C during 0.5 hours, 1 hours, 1.5 hours. Then, determining the percentage of survival by counting colonies on plates [5].
The percentage of survival= colony forming units survive/colony forming units in sample control*100.

2.2.5. Adhesion to epithelial intestine

Culture medium was activated two times in 30 ml MRS broth, centrifuge 4000 rpm/minutes in 15 minutes and collected biomass washed two times in PBS buffer solution (pH 6.5) then dissolved again in 4 ml PBS buffer solution. Adhesion was performed during 5 hours. In zero hour, samples were vortex 10 seconds, took 0.1 ml samples dissolved in 3.9 ml buffer solution and measured OD at 600 nm. At 1, 3, 5 hour, took 0.1 ml samples dissolved in 3.9 ml buffer solution and measured OD at 600 nm [5].
The percentage of adhesion=$(1-A_t/A_0)*100$
$A_{0:}$ OD_{600} measured at 0 hour
A_t: OD_{600} measured at 1, 3, 5 hour

2.2.6. Evaluation of properties probiotic

Antimicrobial activity and resistance antibiotic bacteria
Samples were cultured in MRS broth during 48 hours and incubated in 37^oC. Culture medium were collected by centrifuging, culture medium (suspension) were controlled in pH 6.5 by NaOH and sterilized by filter membrane. Suspension was spread within each well contained tested bacteria (*Escherichia coli A*TTC 25922, *Staphylococcus aureus* ATCC 29213, *Staphylococcus aureus* resist to methicillin (MRSA) and incubated in 37^0C during 48 hours. The antimicrobial activity was assessed by measuring the clear zone that formed around each well [6].
Lactate activity test
10 ml culture medium were centrifuged 5000 rpm/min during 15 minutes in 4^0C, biomass were washed two times in 10 ml buffer solution sodium phosphate 0.1M pH 7 and centrifuged 5000rpm/min during 15 minutes in $4^{0C.}$. Then, biomass were added to 10 ml above buffer solution and vortex strongly to mix solution. Lactate activity was determined by Sriphannam method. Dissolving 1.5 ml ONPG 4mM in buffer solution sodium phosphate 0.1M pH 7. Using OD 420nm to measure ONP produced in the reaction [7].

Table 2.1. Preparation of test samples to determine lactate activity

Medical test	Real samples	Unreal samples
Enzyme solution in buffer solution Na-PP 0.1M	1.5 ml	1.5 ml
Solution Na_2CO_3	0 ml	7 ml
Solution ONPG 4mM in buffer solution Na-PP 0.1M	1.5 ml	1.5 ml
	Incubate in 37^0C during 10 minutes	
Na_2CO_3 0.5M	7 ml	0 ml

Lactate activity= $C_{OPN} \cdot \frac{1}{t} \cdot \frac{V_{hh}}{V_{enzyme}} .a$

CONP: concentration of ONP produced, determined by standard line equation (mM)

t: reaction time (minutes)

V_{hh}: the volume of mixed reaction {ml}

V_{enzyme}: the volume of enzyme reaction (ml)

a: distilled constant

2.2.7. Determination of isolated strains

Isolated probiotic strains were determined by API 50CHL kit and APL software

Potential probiotic strains were determined by 16SrRNA in Nam Khoa company Ho Chi Minh city.

2.2.8. Data analyses

Data were processed by Excel and SPSS.

III. Results And Discussion

3.1. Results of isolation and colony forming units lactic strains

3.1.1. The results and name of isolated lactic strains

A total 7 samples (breast milk) were provided by Hung Vuong hospital and mothers raising babies from 1 to 3 months, isolated 22 lactic strains from normal born (HV2, HV3, HV4, M1, M2, M3) and we did not isolate lactic strains from HV1 (operated born) after 1 day. The isolated lactic strains were selected based on colony morphology, cell morphology. 22 isolated lactic strains were suitable with *Lactobacillus* because they have rod shaped morphology and Gram +. The name and some characteristics of isolated lactic strains were showed in table 3.1.

Table 3.1. Name and phenotype characteristics of isolated strains from breast milk

Group	Sample	Strains	Gram	Morphology cell	Group	Sample	Strain	Gram	Morphology cell
1	HV2	HV2.1	(+)	Rod shape	11	M1	M1.1	(+)	Rod shape
2		HV2.2	(+)	Rod shape	12		M1.2	(+)	Rod shape
3		HV2.3	(+)	Rod shape	13		M1.3	(+)	Rod shape
4		HV2.4	(+)	Rod shape	14		M1.4	(+)	Rod shape
5	HV3	HV3.1	(+)	Rod shape	15	M2	M2.1	(+)	Rod shape
6		HV3.2	(+)	Rod shape	16		M2.2	(+)	Rod shape
7		HV3.3	(+)	Rod shape	17		M2.3	(+)	Rod shape
					18		M2.4	(+)	Rod shape
8	HV4	HV4.1	(+)	Rod shape	19	M3	M3.1	(+)	Rod shape
9		HV4.2	(+)	Rod shape	20		M3.2	(+)	Rod shape
10		HV4.3	(+)	Rod shape	21		M3.3	(+)	Rod shape
					22		M3.4	(+)	Rod shape

Lactic strains in breast milk were affected strongly by normal born or operated born, physiological disorder in operated born is heavier than normal born since lactic strains in breast milk are changed. In operated born, anesthetic affects to the production of milk in mothers; besides, antibiotics prevent infections after operated born also inhibit created milk hormones and lactic strains in breast milk. Babies in operated born do not have useful lactic strains, but they have harmful bacteria such as *Enterococcus* and *Klebsiella.* In contrast; babies in normal born have useful lactic strains such as *Lactobacillus acidophilus* [3].

3.1.2. The colony forming units lactic strains in breast milk

Breast milk is good nutrients for born babies. It provides useful lactic strains help activation of digestive system in born babies. Lactic strains can transfer from gut to breast milk and then transfer to born babies. Results of colony forming units lactic strains in each period of breast milk showed in table 3.2. These results were not processed by statistic because samples were restricted and not enough size samples.

Table 3.2. The colony forming units lactic strains in breast milk

Group	Samples	The colony forming units (log CFU/mL)
1	HV1	0
2	HV2	8,214
3	HV3	8,018
4	HV4	7,987
5	M1	8,417
6	M2	7,948
7	M3	8,110

Lactic strains in breast milk are affected by many factors such as environment, heritage. In this newspaper, we considered the change of colony forming units lactic strains in breast milk in different periods from 2-3 days to 92 days. As a result, lactic strains could not be isolated in HV1 sample; remaining samples contained high colony forming units lactic strains from 7.948 to 8.417 log CFU/ml. According to Rocio Martin (2003), the colony forming units lactic strains in breast milk isolated in MRS medium were 10^5-10^7 (CFU/ml).

In breast milk 2-3 days, there were different colony forming units from 7.948 to 8.417 log CFU/ml among samples. Early breast milk is produced in 2-4 days after babies are born. It contains many vitamins and minerals. The different colony forming units lactic strains is caused by many factors such as environment, geography, sexual babies and mother's nutrients. *Lactobacillus* has the biggest colony forming units in early breast milk [8].

After two weeks, early breast milk becomes to mature breast milk. In this period, the colony forming units lactic strains were the best (8.417 log CFU/ml). According to Pardo (2014), the colony forming units lactic strains in early breast milk were lower than mature breast milk.

3.2. Evaluation of probiotic strains

3.2.1. Tolerance to Acids

The colony forming units lactic strains at 0 hour in pH 2.5 had differences. Most colony forming units of isolated strains decreased after 3 hours. 12/22 isolated strains had ability exist in low pH well, the rate of survival were over 50%; besides, 7 strains had the rate of survival over 90% and the rate of survival of HV4.1 were 100%. According to Wenjun Liu (2020), *B. lactic* Probio-M8 and *L. rhamnosus* Probio-M9 have high rate of survival in pH 2.5 (97.25%, 78.33%). The result of tolerance to acids were showed in table 3.3.

Table 3.3. The rate of survival of isolated strains at 3 hour in culture medium pH 2.5

Group	Samples	The rate of survival (%)	Group	Samples	The rate of survival (%)
1	**HV2.1**	**91,21**	**12**	**M1.2**	**94,53**
2	**HV2.2**	**95,61**	13	M1.3	4,97
3	HV2.3	0,48	14	M1.4	0,94
4	HV2.4	3,29	15	M2.1	0,75
5	**HV3.1**	**96,7**	**16**	**M2.2**	**96,63**
6	**HV3.2**	**72,8**	17	M2.3	0,93
7	**HV3.3**	**54,6**	18	M2.4	0,81
8	**HV4.1**	**100**	19	M3.1	1,39
9	**HV4.2**	**95,5**	**20**	**M3.2**	**75,75**
10	**HV4.3**	**67,8**	21	M3.3	6,76
11	M1.1	3,64	**22**	**M3.4**	**77,57**

Note: Bold strains have high the rate of survival (>50%)

The isolated strains could not tolerance to acids, their rate of survival were very low (<10%). pH 2.5 is harsh condition with microorganism includes lactic strains. The reason is that pH 2.5 inhibits enzyme activities and biochemistry mechanisms in microorganism. Therefore, the rate of survival is low or cannot exist in pH 2.5 [6].

3.2.2. Tolerance bile salt

We used SGJ culture medium added 0.3% bile salt to evaluate tolerance bile salt in isolated lactic strains. The reason is that 0.3% bile salt is normal concentration in human gut. After 3 hours, we evaluated the rate of survival isolated strains and results were showed in table 3.4. There were 8 over 22 isolated strains survived in bile salt medium (>50%) and SGJ medium. In addition; there were 3 over 8 strains survived 100% included HV2.2, HV4.1, M3.2. These results similar to the results of Ramos, Meltem Asan (2018) that performed in *Lactobacillus fermentum* isolated from breast milk.

14 strains (remaining strains) had low the rate of survival in SGJ added bile salt (<50%). In addition; 2 strains could not survive in medium added bile salt included M2.3, M3.1 (<10%). The colony forming units lactic strains decreased quickly after 3 hours; according to Wei Tang (2012), the colony form units lactic strains decreased a half after 3 hours.

Table 3.4. The rate of survival lactic strains at 3 hour in SGJ medium added 0.3% bile salt

Group	Samples	The rate of survival (%)	Group	Samples	The rate of survival (%)
1	HV2.1	33,26	**12**	**M1.2**	**50,69**
2	**HV2.2**	**100**	13	M1.3	32,81
3	HV2.3	4,19	14	M1.4	16,21
4	HV2.4	39,54	15	M2.1	32,14
5	**HV3.1**	**94,5**	**16**	**M2.2**	**63,38**
6	**HV3.2**	**71,2**	17	M2.3	9,26
7	HV3.3	49,7	18	M2.4	31,40
8	**HV4.1**	**100**	19	M3.1	4,49
9	**HV4.2**	**94,1**	**20**	**M3.2**	**100**
10	HV4.3	46,2	**21**	M3.3	24,27
11	M1.1	47,09	22	M3.4	12,02

Note: bold strains have high rate of survival (>50%)

3.2.3. Adhesion to epithelium intestine

Adhesion to epithelium intestine helps lactic strains survive and develop well in digest system. In addition; adhesion helps to prevent harmful bacteria attack digest system. The adhesion of isolated strains were showed in table 3.5.

According to Rajoka (2017), isolated strains have ability adhere to epithelium intestine well after 5 hours (>40%). HV4.1 strain had the best adhesion after 5 hours (96.2%). M3.1 strain had the lowest adhesion after 5 hours (55.9%). According to Rajoka (2017), adhesion of *Lactobacillus rhamnosus* isolated from breast milk over 30%, all isolated strains were high adhesion after 24 hours (>50%). [6].

Table 3.5. Adhesion of isolated lactic strains

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Group	Samples	The percent of adhesion (%)		
		After 1 hour	After 3 hour	After 5 hour
1	HV2.1	12.3	45.6	75.2
2	HV2.2	13.1	41.5	80.5
3	HV2.3	10.5	39.5	69.6
4	HV2.4	14.2	44.5	84.9
5	HV3.1	12.5	42.9	90.6
6	HV3.2	12.1	42.1	87.3
7	HV3.3	11.5	51.4	81.9
8	HV4.1	14.2	58.5	96.2
9	HV4.2	12.8	51.8	91.7
10	HV4.3	14.1	54.0	94.0
11	M1.1	13.5	47.8	87.7
12	M1.2	12.2	52.0	82.9
13	M1.3	12.5	52.1	82.2
14	M1.4	12.1	59.1	96.1
15	M2.1	11.5	51.4	91.8
16	M2.2	13.5	43.9	73.0
17	M2.3	12.4	42.7	62.8
18	M2.4	13.1	45.6	65.0
19	**M3.1**	**13.5**	**42.6**	**55.9**
20	M3.2	12.2	42.5	62.2
21	M3.3	10.6	40.8	60.2
22	M3.4	14.2	44.6	64.1

Note: Bold strain has the lowest adhesion

After evaluating probiotic activities such as surviving in harsh conditions in digest system, adhering of lactic strains. We continue to evaluate probiotic properties in isolated lactic strains.

3.3. Evaluation of probiotic properties in isolated lactic strains

3.3.1. Antimicrobial and antibiotic susceptible activity

Lactic strains move from stomach to small intestine, lactic strains express their probiotic properties such as resisting to harmful bacteria. We evaluated antimicrobial activity in 22 isolated strains with two tested bacteria (*Staphylococcus aureus* ATCC 29213 and *Escherichia coli* ATCC 25922), antibiotic susceptible activity with tested bacteria *Staphylococcus aureus* (MRSA). Lactic strains inhibit harmful bacteria cause gut disease by producing organic acids decrease pH and antimicrobial substances. Isolated strains have high antimicrobial activity when they have large clear zone.

Antimicrobial activity was evaluated by using Agar well diffusion method, where antimicrobial activity was determined by measuring the clear zone . After 16 hours, the clear zone was formed around each disc, the result was showed in table 3.6.

The result showed that isolated strains have ability resist three tested bacteria. Most isolated strains have average resistance to *S.aureus*, the clear zone was from 3-14 mm. This result similar to Parisa Shokryzdan's research (2014), *L.acidophilus* and *L.fermentum* resisted to *S.aureus*, the clear zone were 6.7 mm and 14,5 mm.

The isolated lactic strains had average resistance to MRSA and *E.coli*, the clear zone MRSA were from 7.6-12.5 mm, the clear zone *E.coli* were from 8.3 to 12.3 mm. The result similar to Ashraf's research (2014), the clear zone diameter of *L.salivarius*, *L.acidophilus* with *E.coli were* 6.8 mm and 8 mm.

Table 3.6. The result of antibacteria activity

Group	Samples	*S.aureus* ATCC 29213		MRSA		*E.coli* ATCC 25922		Note
		The clear zone (mm)	Sensitive	The clear zone (mm)	Sensitive	The clear zone (mm)	Sensitive	
1	HV2.1	6,0	(**)	10,1	(**)	9,4	(**)	
2	HV2.2	5,0	(*)	10,5	(**)	10,1	(**)	
3	**HV2.3**	3,0	(*)	**12,5**	(**)	**12,3**	(**)	
4	HV2.4	6,0	(**)	10,3	(**)	9,2	(**)	
5	HV3.1	5,8	(*)	7,6	(**)	9,8	(**)	
6	HV3.2	9,6	(**)	9,1	(**)	8,6	(**)	
7	HV3.3	10,5	(**)	10,2	(**)	10,8	(**)	
8	HV4.1	12,4	(**)	11,5	(**)	11,9	(**)	
9	HV4.2	12,1	(**)	11,8	(**)	11,1	(**)	
10	HV4.3	11,5	(**)	10,9	(**)	11,6	(**)	
11	M1.1	5,0	(*)	10,4	(**)	9,3	(**)	
12	M1.2	5.0	(*)	12,4	(**)	11,1	(**)	
13	M1.3	10,0	(**)	11,5	(**)	10,2	(**)	
14	M1.4	9,0	(**)	9,0	(**)	9,2	(**)	
15	M2.1	10,0	(**)	10,8	(**)	10,1	(**)	
16	M2.2	5,0	(*)	10,5	(**)	10,2	(**)	
17	**M2.3**	13,0	(**)	10,9	(**)	**12,3**	(**)	
18	M2.4	10,0	(**)	11,0	(**)	10,3	(**)	
19	M3.1	4,0	(*)	10,1	(**)	8,8	(**)	
20	**M3.2**	**14,0**	(**)	9,5	(**)	8,5	(**)	
21	M3.3	12,0	(**)	11,6	(**)	8,3	(**)	
22	M3.4	8,0	(**)	9,0	(**)	9,2	(**)	

Note: (***): strong antibacterial activity (diameter >15 mm)

**: the average antibacterial activity (6 mm<diameter<15 mm)

*: weak antibacterial activity (1 mm≤ diameter ≤ 6 mm)

S.aureus resist to methicillin (MRSA) and cause infection in the hospital. According to Hanna Sikorska, *L. reuteri* and *L. rhamnosus* GG resist to *S.aureus*; besides, *L.acidophilus* also inhibit the growth of *S.aureus* (MRSA) by creating biofilm.

The resistance of M 3.2 strain with *S.aureus* were the strongest antibacterial activity (diameter = 14 mm), the resistance of HV 2.3 with MRSA were the strongest (diameter = 12.5 mm). M 3,2 and HV 2.3 strains resist to *Ecoli* well (diameter = 12.3 mm).

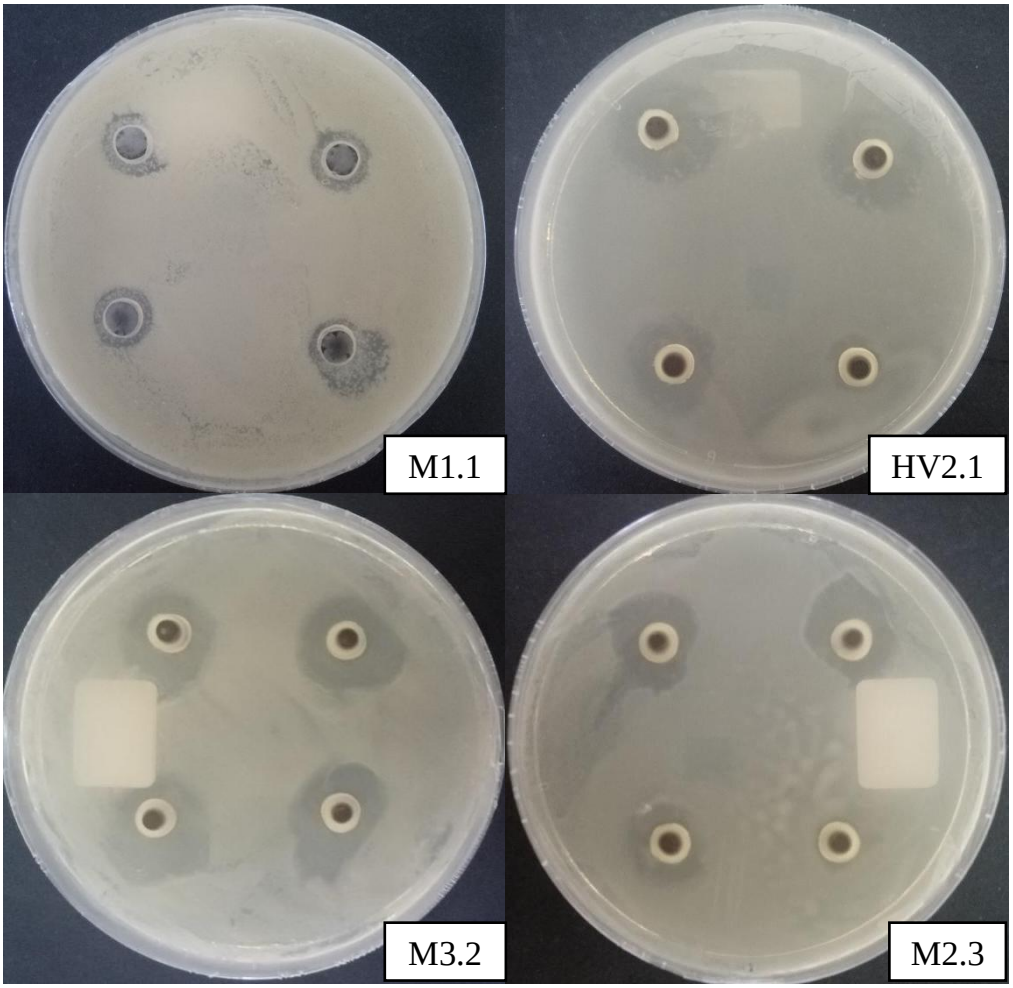


Figure 3.1: The picture of isolated strains resist to *S.aureus*

3.3.2. Evaluation of lactate activity in isolated strains

We evaluated lactate activity in 22 isolated strains and showed in table 3.7. As a result; lactate activity in isolated strains were higher than normal probiotic strains such as *L. acidophilus* ATCC 4356 (0,12 U/ml), *L. casei* ATCC 334 (0,09 U/ml). The lactate activity in HV 4.1 were the best (0.58 U/ml), the lactate activity in M 3.3 were the lowest (0.25 U/ml). There were 8 strains expressed potential lactate activity (> 0.4 U/ml) HV2.1, HV2.2, HV3.2, HV3.3, HV4.1, HV4.2, HV4.3, M1.1. Theses 22 strains were isolated from breast milk (2-3 days after born).

Table 3.7. The lactate activity of isolated strains

Group	Samples	Lactate activity (U/mL)	Group	Samples	Lactate activity (U/mL)
1	**HV2.1**	**0,40**	12	M1.2	0,33
2	**HV2.2**	**0,51**	13	M1.3	0,37
3	HV2.3	0,39	14	M1.4	0,29
4	HV2.4	0,35	15	M2.1	0,35
5	HV3.1	0,38	16	M2.2	0,30
6	**HV3.2**	**0,42**	17	M2.3	0,35
7	**HV3.3**	**0,46**	18	M2.4	0,39
8	**HV4.1**	**0,58**	19	M3.1	0,30
9	**HV4.2**	**0,51**	20	M3.2	0,28
10	**HV4.3**	**0,48**	21	M3.3	0,25
11	**M1.1**	**0,42**	22	M3.4	0,32

According to some research; lactic strains produce lactate to digest lactose in milk. Some strains are *L.bulgaricus* (0.418 U/ml), *L.acidophilus* AS186 (0,218 U/ml), *L.casei* VTCC (0,1825 U/ml) [14]. Babies did not have bacteria in their intestine during 24 hours after born, but the colony forming units bacteria developed well at 3-day. The reason is that lactic strains in breast milk stimulated the growth of bacteria in intestine and help babies digest breast milk. Therefore, breast milk were considered potential sources produce lactate. As a result; the production lactate of lactic strains in breast milk were higher than strains were isolated from fermented food.

3.4. Determination of isolated strains

3.4.1. Determination of isolated strains by kit API

22 lactic strains were isolated from breast milk; then, we determined isolated strains by kit API. The result were showed in table 3.8

Table 3.8. The results of determined 22 isolated strains

Group	Samples	Determination	Group	Samples	Determination
1	HV2.1	*Lactobacillus casei*	12	M1.2	*Lactobacillus paracasei*
2	HV2.2	*Lactobacillus paracasei*	13	M1.3	*Pediococcus lolii*
3	HV2.3	*Lactobacillus acidophilus*	14	M1.4	*Lactobacillus pentosus*
4	HV2.4	*Lactobacillus acidophilus*	15	M2.1	*Lactobacillus kefiri*
5	HV3.1	*Lactobacillus fermentum*	16	M2.2	*Lactobacillus brevi*
6	HV3.2	*Lactobacillus paracasei*	17	M2.3	*Lactobacillus paracasei*
7	HV3.3	*Lactobacillus acidophilus*	18	M2.4	*Lactobacillus acidophilus*
8	HV4.1	*Lactobacillus paracasei*	19	M3.1	*Lactobacillus casei*
9	HV4.2	*Lactobacillus acidophilus*	20	M3.2	*Lactobacillus rhamnosus*
10	HV4.3	*Lactobacillus brevi*	21	M3.3	*Lactobacillus acidophilus*
11	M1.1	*Lactobacillus plantarum*	22	M3.4	*Lactobacillus fermentum*

3.4.2. Evaluation of lactic strains isolated from breast milk, selection and determination of potential strains by 16S rDNA

3.4.2.1. Evaluation of lactic strains isolated from breast milk

After evaluating probiotic activities and probiotic properties, we summarized details and the result was showed in table 3.9

Table 3.9. Evaluation of probiotic activities in isolated strains

Group		Samples	pH	Bile salt	Adhesive activity	Antibacteria	Lactate activity
1	HV2.1	*Lactobacillus casei*	+	-	+	+	+
2	HV2.2	*Lactobacillus paracasei*	+	+	+	-	+
3	HV2.3	*Lactobacillus acidophilus*	-	-	+	-	+
4	HV2.4	*Lactobacillus acidophilus*	-	-	+	+	+
5	HV3.1	*Lactobacillus fermentum*	+	+	+	-	+
6	HV3.2	*Lactobacillus paracasei*	**+**	**+**	**+**	**+**	**+**
7	HV3.3	*Lactobacillus acidophilus*	+	-	+	+	+
8	HV4.1	*Lactobacillus paracasei*	**+**	**+**	**+**	+	+
9	HV4.2	*Lactobacillus acidophilus*	+	+	+	+	+
10	HV4.3	*Lactobacillus brevi*	+	-	+	+	+
11	M1.1	*Lactobacillus plantarum*	-	-	+	-	+
12	M1.2	*Lactobacillus paracasei*	+	+	+	-	+
13	M1.3	*Pediococcus lolii*	-	-	+	+	+
14	M1.4	*Lactobacillus pentosus*	-	-	+	+	+
15	M2.1	*Lactobacillus kefiri*	-	-	+	+	+
16	M2.2	*Lactobacillus brevi*	+	+	+	-	+
17	M2.3	*Lactobacillus paracasei*	-	-	+	+	+
18	M2.4	*Lactobacillus acidophilus*	-	-	+	+	+
19	M3.1	*Lactobacillus casei*	-	-	+	-	+
20	M3.2	*Lactobacillus rhamnosus*	+	+	+	+	+
21	M3.3	*Lactobacillus acidophilus*	-	-	+	+	+
22	M3.4	*Lactobacillus fermentum*	+	-	+	+	+

Note: + : strong activity

-: weak activity

3.4.2.2. Selection of potential strains from isolated strains in breast milk

Individual probiotic activity: Strain has the best activity in each probiotic activity

- ✓ Basic probiotic activity were the best: *L. paracasei* HV4.1
- ✓ Antibacterial activity: *L. paracasei* HV4.1
- ✓ Activity of lactate production: *L.paracasei* HV4.1

Various probiotic activities : one strain contains many probiotic properties

L.paracasei HV4.1 survived well in harsh conditions in digest system such as stomach, bile salt; besides, their adhesion were the best in isolated strains from breast milk. In addition; *L.paracasei* HV4.1 had high antibacterial and lactate activities. Thus; *L.paracasei* HV4.1 were considered strain contains various probiotic activities.

Moreover; 3 strains includes *L.paracasei* HV3.2, *L.acidophilus* HV4.2, *L.rhamnosus* M3.2 considered various probiotic activities. They survived in harsh conditions such as low pH, bile salt and their adhesive activity were high. Moreover; *L.paracasei* HV3.2 and *L.acidophilus* HV4.2 had high antibacterial and lactate activities. Therefore, these 3 strains considered strains contain various probiotic activities.

We compared various probiotic strains and normal probiotic strains; the result was showed in table 3.10

Table 3.10. Compare probiotic activity of potential strains with normal strains

Samples	The percentage of survival in SGJ pH 2.5 (%)	The percentage of survival in SGJ pH 2.5 added bile salt (%)	Adhesive activity (%)	Diameter of clear zone (mm)			Lactate activity (U/mL)
				S. aureus	*E. coli*	MRSA	
L. paracasei HV3.2	72,8	71,2	87,3	9,6	8,6	9,1	0,42
***L. paracasei* HV4.1**	**100**	**100**	**96,2**	12,4	**11,9**	11,5	**0,58**
L. acidophilus HV4.2	95,5	94,1	91,7	12,1	11,1	**11,8**	0,51
L. rhamnosus M3.2	75,75	100	62,2	**14,0**	8,5	9,5	0,28
L. acidophilus ATCC 4356	89,54	100	52,67	**12,25**	**10,55**	**11,9**	0,12
L. plantarum ATCC 14817	98,25	100	55,31	7,42	5,49	7,5	**0,42**
L. casei ATCC 334	91,67	100	61,89	10,61	7,24	10,1	0,09

According to the table 3.10; potential strains have basic probiotic activities and properties similar to normal strains such as surviving in harsh conditions such as low pH, bile salt, adhesive activity, antibacterial activity. Besides; potential strains have lactate activity higher than normal strains. In conclusion; we selected 4 potential strains (HV3.2, HV4.1, HV4.2, M3.2) and determined them by 16S rDNA.

3.4.2.3. Determination of potential strains by 16S rDNA

HV3.2, HV4.1, HV4.2, M3.2 strains were determined in Nam Khoa company. HV3.2 was *Lactobacillus paracasei*, HV4.1 was *Lactobacillus paracasei*, HV4.2 was *Lactobacillus acidophilus*, M3.2 was *Lactobacillus rhamnosus*.

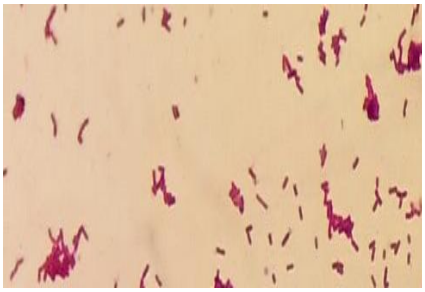
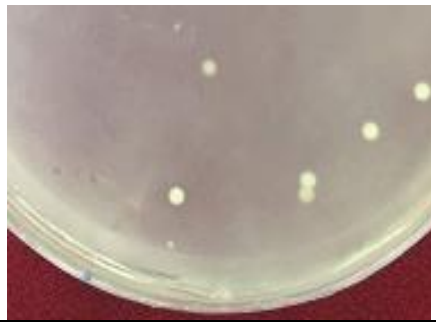
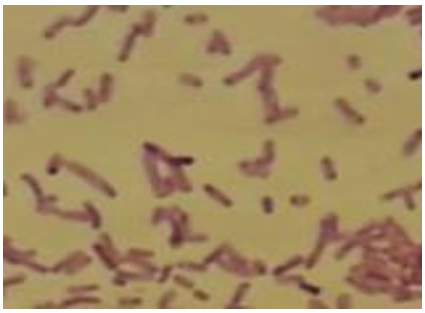
Lactobacillus paracasei were applied in fermented milk, they are very useful for human's digest system. *Lactobacillus paracasei* can inhibit *H.pylori* caused ulcer stomach. In addition; they can exist in high temperature, degrade protein and increase immune system.

Lactobacillus acidophilus produce antibiotic such as bacteriocin, acidophilin that inhibit the growth of harmful bacteria (*Campylobacter*, *Staphylococcus*). Besides, they have ability convert lactose into glucose, prevent cancer disease and decrease cholesterol.

Lactobacillus rhamnosus were isolated from human's intestine; they decrease diarrhea in babies. *Lactobacillus rhamnosus* increase immune system to prevent infection by harmful bacteria; they decrease cholesterol and control glucose level in blood to prevent diabetes in human [13].

Table 3.11. The picture colony and microscope of 4 selected strains

Group	Samples	Colony	Microscope
1	*Lactobacillus paracasei* HV3.2		

2	*Lactobacillus paracasei* HV4.1		
3	*Lactobacillus acidophilus* HV4.2		
4	*Lactobacillus rhamnosus* M3.2		

V. Conclusion

- The colony forming units of isolated strains (except operated born) are high from 7.948 to 8.417 log CFU/ml.
- 22 probiotic strains were isolated from 7 samples (breast milk), they were determined by kit AIP included *Lactobacillus acidophilus*, *Lactobacillus paracasei*, *Lactobacillus casei*, *Lactobacillus fermentum*, *Lactobacillus brevi*, *Lactobacillus plantarum*, *Lactobacillus pentosus*, *Lactobacillus kefiri*, *Lactobacillus rhamnosus* và *Pediococcus lolii*.
- 8 over 22 isolated strains survived in harsh conditions such as stomach, bile salt. In summary; isolated strains resist to the bacteria well (diameter clear zone were form 6 mm-15 mm). Moreover; isolated strains had adhesive and lactate activities that were very high.
- First steps try to create Bio-product from isolated lactic strains in breast milk; its aim can support babies cannot drink breast milk. We selected 4 strains with various probiotic properties included *Lactobacillus paracasei* HV3.2*; Lactobacillus paracasei* HV4.1*; Lactobacillus acidophilus* HV4.2 và *Lactobacillus rhamnosus* M3.2.

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