Available online www.jocpr.com

Journal of Chemical and Pharmaceutical Research, 2016, 8(7):368-372

Research Article

ISSN : 0975-7384
CODEN(USA) : JCPRC5

The effects of hyaluronic acid on the morphological physiological differentiation of *Lactobacillus*

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ABSTRACT

Hyaluronan (HA) molecules are long, unbranched chains of variable length that consist of repeating disaccharide subunit of D-glucuronic acid (b1->3) and N-acetyl-D-glucosamine (b1->4). Owing to its stable properties and high viscosity, hyaluronic acid that derivatives are widely used in food, cosmetics, and pharmaceutical industries. However, the role of HA in probiotics was not known while the probiotics are used very commonly and the number of probiotics are usually lower than the number in label. Therefore, to understand the HA function clearly and look for the activity on probiotics, the research about the effect of HA on the the morphological and physiological differentiation of Lactobacillus was performed. The examination tests were performed by using the light microscope, scanning electron microscope. The bacteriocin tests were done by agar diffusion test. In order to looking for the survival, the colony counting was done. Besides, the experiment used the API 50 CHL kit (BioMerieux) to do biochemical test and identify the strain after treated with hyaluronic acid. The result showed the effects of HA on Lactobacillus in the shape changing, cell growth, bacteriocin production but not effect on the biochemical tests. Even there was the changing in shape, cell growth, bacteriocin production, the experimental L. acidophilus LA1 was still such identified by using API 50 CHL kit (BioMerieux).

Keywords*:* bacteriocin, hyaluronan, *Lactobacillus acidophilus*, morphological and physiological differentiation

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INTRODUCTION

Probiotics are living microorganisms having a positive impact to the host by helping balance the intestinal flora. Bacteria probiotics help the digestion improvement, and kill some pathogenic bacteria through antimicrobial substances (acids, bacteriocins, undigested lactose in milk, reduced diarrhea without causing the harm to the host. Probiotics are now widely used in foods, pharmaceuticals, in animal husbandry. There are a lot of bacteria screened but the predominant species used as probiotic agents belong to the group of lactic acid bacteria (LAB). Within LAB group, the *Lactobacilli* genus is the most widely encountered for probiotics [1] including *L. acidophilus*, *L. bulgaricus*, *L. casei*, *L. fermentum*, *L. plantarum*, *L. reuteri*, *L. rhamnosus*, and *L. salivarius*. Up to date, questions regarding to the reliability, quality of those commercial products as well as safety issues have been raised. There were a lot of researches about the improvement or development of the quality of probiotic products. However, the study about changing the shape, the cell surface and physiological activity of LAB that affect on the quality of LAB were not focused.

Besides, a high molecular mass polysaccharide, hyaluronic acid (hyaluronan, HA) is used in pharmaceutical, cosmetics and food area. HA also plays an important role in cancer metastasis and monitoring the growth of cancer cells. Some of researches tried to create the hyaluronan receptor such as CD44 and RHAMMAl [2], [3]. Recently, there are a number of studies on the influence of HA on microorganisms. It affects on the division process in the growth of microorganisms and alteration of some functions of the organism such as *E. coli* [4].

Based on the properties of hyaluronic acid (HA) with many uses in food and medicines, we hypothesized the effects of hyaluronic acid (HA) on the morphological and physiological differentiation of LAB. Hyaluronic acid is as the new factor supplied in the growth medium for LAB to hopefully improve the characteristics of LAB such as the bacteriocin production, survival, surface attachment and resistant gene transfer between LAB and pathogens.

This was the first report in the study of HA on the cell growth of gram positive, non-motile LAB. The study will drive more knowledge in using HA and probiotics efficiently.

EXPERIMENTAL SECTION

Microorganisms and growth condition

The microorganism *Lactobacillus acidophilus* was used and maintained in MRS agar and MRS broth. The HA concentrations were used as 0.03%, 0.05%, 0.08%, 0.1%. The incubation were performed at 25 - 30^{o}C for 3-4 days.

Quantitative assay

Plate diluting method was applied for quantitative CFU counts (CFU-colony forming units) for determination of respective groups of microorganisms in 1 g of substrate. In pharmaceutical field, the quantification of microorganisms was determined basing on the average of viable cells in 10 g substrate (1:10).

Isolation of bacterial strain and culture conditions

The methods of isolation and cultivation were according to Schillinger [5], using de Man Rosa Sharpe (MRS) agar or broth (Merck, Darmstadt, Germany) as media. A portion of 10g of sample was mixed with 90 ml normal saline to get ratio 1/10. The solution was shaken 10 min to homogenize all of insoluble substrates. A volume of 1 ml of the dilutions was plated on MRS agar and incubated in anaerobic conditions at 30^{o}C for 48 hours. The colonies were tested for *Lactobacilli* by microscopic examination with gram stain and catalase production to observe the mobility, shape, size and color properties. The gram positive, catalase-negative rods were selected for the studies. The isolated strains were identified by biochemical characterization based on the ability of the isolates to utilize or oxidize different carbon sources, which determined by API 50 CHL systems (bioMérieux, Lyon, France).

Scanning electron microscope (SEM)

After cultured, 1ml sample was centrifuged with 10000 rpm in 10 minutes to collect pellet then dehydrated by absolute ethanol. Finally, the samples were stained with platinum before observation.

Bacteriocin tests

The test was performed by using agar disc diffusion. The indicative strains were Bacillus subtilis and Bacillus cereus. After the incubation, the diameters of the inhibition zones were measured.

RESULTS AND DISCUSSION

Morphological observation

The HA was add into the MRS broth in 5 tubes to obtain the final concentrations as 0, 0.03%, 0.05%, 0.08%, 0.1%. After 3-4 days of incubation, *L. acidophilus* grew well in the experimental conditions. Figure 1 showed the shapes of *L. acidophilus* after incubation in HA. In medium with 0.03%, 0.05% of HA, the cell was shorter than in the medium without HA. In medium with 0.03% HA, the cell is shortest. In medium with 0.08%, 0.1% of HA, the cell become longer than in the medium without HA.

Bacteriocin activities

The samples were taken to test the ability of bacteriocin producing by using the agar disc diffusion method. Each disc contained 30µl of sample was placed on the LB agar disseminated 200 µl of *Bacillus cereus* or *Bacillus subtilis*. By measuring the zone diameter, the activities of L. acidophilus on *Bacillus cereus* or *Bacillus subtilis* after being treated by HA are stronger than *L. acidophilus* produced in medium without HA. The results were presented in the table 1.

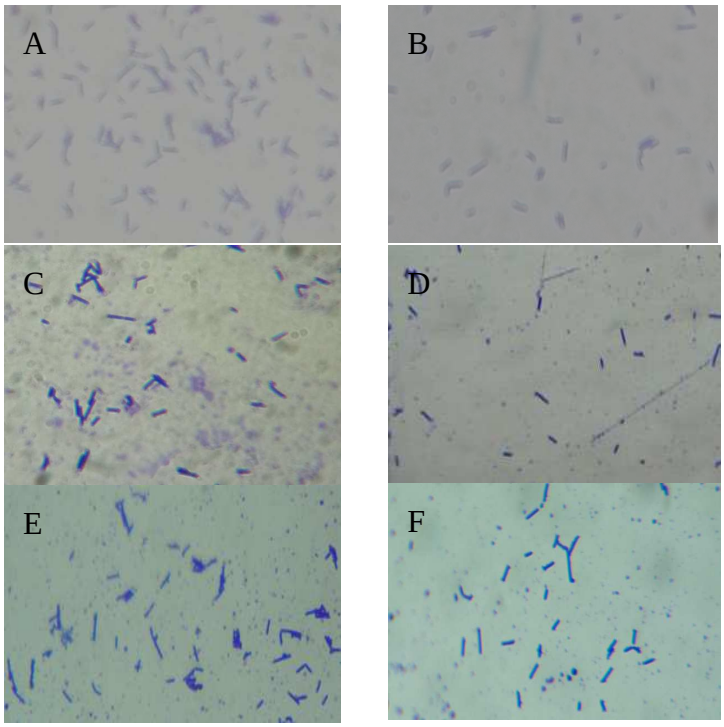

Figure 1: The gram stainning of *L. acidophilus* with different HA concentration
A: 0% ; B: 0.02%; C: 0.03% ; D: 0.05%; E: 0.08% ; F: 0.1%

Table 1: The bacteriocin test results

Concentrations of HA(%)	Bacillus subtilis (diameter zones{cm})	Bacillus cereus (diameter zones{cm})
0	0.9	1.1
0.03	1.1	1.35
0.05	1.25	1.4
0.08	1.3	1.6
0.1	1.3	1.6
	$\rho<0.05$	$\rho<0.05$

Effects of HA on survival of *L. acidophilus*

In this experiment each sample corresponding to HA concentration was done in three replicates with 2 dilution factors 10^4, 10^6. The result can be received after 15, 28 days. With dilution factor 10^4, there were too many colonies grown in the medium agar and data was not shown. In the dilution factor 10^6, the results were shown in table 2 and table 3.

Table 2: The quality of L. Acidophilus after 15 days in 10^6 dilution

HA concentration (%)	$x10^6$/50 (µl) (number of colonies in three replicates)	Average ($x10^6$/50 (µl))	$x10^9$(CFU/ml)
0	94:80:86	86.67	1.73
0.03	88:80:88	85.3	1.72
0.05	94:88:88	90	1.8
0.08	94:88:96	92.67	1.85
0.1	100:110:96	102	2.04

CFU/ mL = CFU/plate x dilution factor x 1/aliquot

Table 3: The quality of *L. acidophilus* after 15 days in 10^6 dilution

HA concentration (%)	$x10^6$/20 (µl) (number of colonies in three replicates)	Average $x10^6$/20 (µl)	$x10^8$(CFU/ml)
0	7;6;7	6.3	3.15
0.03	8:7:9	8	4
0.05	9:8:9	8.6	4.3
0.08	8;7;9	8	4
0.1	10:11:10	10.3	5.15

Biochemical effects of HA

By using of API 50 CHL kit containing 50 kinds of chemicals, the biochemical tests were done after 24 hours and 48 hours. Under HA treatment, the biochemical properties of *L. acidophilus* were not changed.

The SEM observation

The SEM results were observed as in figure 2.

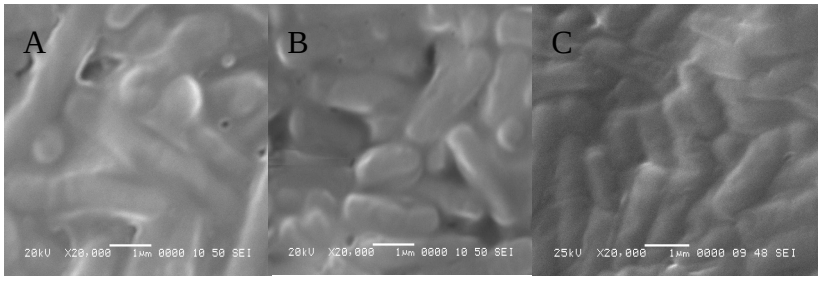

Figure 2: *L. acidophilus* with HA (A: 0%; B: 0.05%; C: 0.1%) SEM

By using the gram stain and scanning electron microscope, the differentiation of L. acidophilus LA1 in morphology has been affected by HA (Figure 1). With the 0.03 % -0.05 % of HA, the cell shape was shorter than in medium without HA. Remarkably, the cell shape in medium with 0.08 % -0.1 % of HA was longer than in medium without HA (Figure 1 C; D; E; F). The reasons of the clear morphological differentiation may be the HA utilization or the HA effects on the cell division. With the 0.03 % and 0.05 % of HA, HA might be affect on the early stage of cell growth of *L. acidophilus*. In the other hands, under the effects of 0.08 % and 0.1 % of HA, the cell was longer than the normal. This might be the use of HA after the microorganism had the short shape in early stage. The effects of HA in the early stage of microorganism have been reported in motile, gram negative *E. coli* [4]. Because this was the first examination in the non-motile, gram positive *L. acidophilus* and the tools for study this field were not equipped to give the percentage of the cells changing the shape and the time lapse of cell division were not measured.

When using HA the untreated and treated *L. acidophilus* to test the bacteriocin activities, the inhibition zones have the larger sizes than the inhibition zone created by the wide-type *L. acidophilus* (Table 1). This results might be caused by the changing the structure in the cell wall of *Lactobacillus acidophilus* [6]. Comparison of the activites of bacteriocin from treated and non-treated *L. acidophilus*, HA might be effect on the cell wall. With the scanning electron microsope, the surface of cell wall was affected (Figure 2).

Besides, the results obtained from biochemical tests of the sample after treated with hyaluronic acid demonstrated that the HA to have not biochemical effects on *L. acidophilus*. By using the API 50 CHL kit supplied by Biomeriux and API software to analyze the score, the sample still pointed that the HA treated microorganism was *L. acidophilus* LA1. The reliability of this method is 99% with general lactobacillus acidophilus [7]. The survival testing of *L. acidophilus* after 15, 25 days that based on the number of colony forming unit (CFU) was shown in the table 2 and 3. The results pointed that HA is safe for *L. acidophilus* without causing death. Consequently, we could evaluate HA did not alter the characteristics and biological functions of *L. acidophilus*. Therefore, with the effects of HA, the test for attachment in the cell line will be performed. With the initial study about the interaction of HA and probiotics, the improvement about the quality of probiotics and the increasing of the biological product producing from probiotics will be obtained.

Probiotics produce an array of microbial defense systems. These include classical antibiotics, metabolic by-products, lytic agents, numerous types of protein exotoxins, and bacteriocins (the protein antibiotics). Bacteriocins are the most abundant of a range of antimicrobial compounds facultatively produced by bacteria, and are found in all major bacterial lineages. These novel substances have been described as extracellular macromolecular protein/peptide antibiotics produced by certain bacteria which exert their lethal effect(s) on bacteria of the same or the related groups. Hyaluronic acid increased the bacteriocin excretion that will be a promising factor in medicine, food and cosmetics. The morphological differentiation in *L. acidophilus* caused by HA will be an option to study the cell division in *L. acidophilus*. Although only a preliminary test the effect of HA on the probiotic product, the results will open the new directions for finding a new formulation for the manufacture of biological products. HA may be expected as a carbon source for new probiotic providing the better improvement in terms of structure and biological function. In addition, these results helps us to properly assess people are using probiotic products and hyaluronic acid on the market, especially the exits of the probiotic which can synthesis hyaluronic acid used in daily probiotic preparation. More studies will be performed so far.

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