

Original Research Article

<https://doi.org/10.20546/ijcmas.2019.809.329>

Isolation and Characterization of Indigenous Probiotic Bacteria from Silkworm (*Bombyx mori* L.) Gut

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ABSTRACT

Keywords

Silkworm gut samples, culture and biochemical characteristics and lactic acid bacteria

Article Info

Accepted:

24 August 2019

Available Online:

10 September 2019

Lactic acid bacteria are described as heterogeneous group of regular, gram-positive, rod shaped, non-motile, non-spore forming bacteria with absence of catalase enzyme. Laboratory study was undertaken to isolate and characterize the lactic acid bacteria from silkworm gut in phenotype basis. A total of 21 silkworm gut samples were collected from silkworm breeds, Double hybrid {(CSR 6 X CSR 26) X (CSR 2 X CSR 27)} obtained from Chawki rearing center (CRC) at Annur, Coimbatore. The bacteria samples were isolated after growth on MRS agar medium and pure culture was obtained by sub-culturing on the same medium. Purity of each culture was confirmed by morphological investigation, Gram's staining and further identification were done by lactic acid bacteria through specific biochemical tests. Finally eight bacterial isolates (SWGL 6, SWGL 7, SWGL 8, SWGL 9, SWGL 10, SWGL 11, SWGL 23 and SWGL 30) were identified as lactic acid bacteria group after morphological, cultural and biochemical characterization.

Introduction

Probiotics are live microbial food supplements used to be beneficial to the host by improving the microbial balance (Fuller, 1991 and Austin *et al.*, 1995). Lactic acid bacteria (LAB) are group of Gram-positive bacteria that are devoid of cytochromes, prefer anaerobic conditions, fastidious, acid-tolerant and strictly fermentative. They are usually non-motile and non-sporulating bacteria that produce lactic acid. Several researchers have reported about beneficial

role of probiotic in humans (Chan *et al.*, 1985; Douillet and Langton, 1994; Gildberg *et al.*, 1997; Brigidi *et al.*, (2000) and insects (Dillon and Dillon, 2004). Therefore, products containing probiotic bacteria are gaining popularity, increasing their importance. Presence of different types of bacteria in the gut has been already reported in mulberry silkworm (*Bombyx mori*) (Roy *et al.*, 2000 and Kodama, 2001).

Metchnikoff (1907) first implied that ingested bacteria in the form of fermented foods could

beneficially affect the normal gut flora. These properties include the beneficial influences that probiotic apparently exert on the microbial ecology of the host (Salminen, 1998). The physicochemical conditions that persuade the composition of the intestinal microbiota include intestinal motility, pH, redox potential, nutrient materials, host secretions (e.g. digestive enzymes, hydrochloric acid, bile and mucus), and the presence of an intact ileocaecal valve (Booijink, 2007). The gastrointestinal (GI) tract harbors many distinct niches, with diverse microbial ecosystem that increases along the GI tract (Gerritsen, 2011). Apart from supporting the digestive functions, indigenous microbes also act as a main actor of the immune system by preventing the colonization by pathogenic microorganisms. Hence, present study was undertaken to isolate and characterize the lactic acid bacteria from silkworm gut samples on the basis of their phenotypic characteristics.

Materials and Methods

Collection of sample

Healthy mulberry leaves of variety, V-1 were fed, Double hybrid {(CSR 6 X CSR 26) X (CSR 2 X CSR 27)} breed was obtained from Chawki rearing center (CRC) at Annur, Coimbatore. Rearing was done under hygienic conditions with optimum temperature (25-28°C) and relative humidity (60-70%). The mulberry leaves were washed thoroughly in running water, shade dried and surface sterilized under UV light before given as feed to silkworms. Leaf feeding was given 3-4 times a day after hatching. Two day old healthy fifth instar larvae were selected and kept under starvation up to 12-16 hours for isolation and enumeration of endogenous gut micro flora. The larvae were anesthetized with 70 per cent chloroform, surface sterilized with 70 per cent alcohol and alimentary canal

was dissected out and transferred to water blank. After isolation, the gut was homogenized immediately in 0.1 M potassium phosphate buffer (pH 7.0), washed with glutaraldehyde-free phosphate buffer and preserved at 5°C (Breznak, 2000).

Isolation of bacteria

Isolation was done by using serial dilution method. Serial dilutions of gut samples were prepared and then sample from different dilutions (10^6 to 10^8) were spread over the solidified MRS (de Man, Rogosa, and Sharpe) medium for isolation. Plates were then incubated at 37°C for 24-48 hrs. After incubation isolated colonies were restreaked on MRS agar plate and pure cultures were isolated.

Characterization of isolates

All the pure cultures samples isolated from silkworm gut were studied for their morphological, cultural and some biochemical characteristics.

Morphological examination

Morphological examination was carried out by examining colony morphology characteristics of all the isolates.

Culture and biochemical characterization

Cultural and biochemical characterization of test isolates were done by Gram (1984) staining, motility test, oxidase test, endospore test, catalase and sugar fermentation test. For motility test, sterilized inoculating needles with culture were stab cultured to 2/3 of the way down to the bottom of test tubes containing motility medium. The tubes were incubated at room temperature for 24-48 hrs and observed for the presence or absence of growth along the line of the stab inoculation.

For endospore test, bacterial smear was aseptically heat fixed on microscopic slide. The slide was placed over steaming water bath and malachite green stain was added for 5 min. The slide was removed and rinsed with tap water. Counter stain safranin was applied for 30 seconds and rinsed with water. Blot dried slides were observed under light microscope for the presence of endospores.

For catalase test, a drop of hydrogen peroxide was taken on a clean glass slide and culture was picked with sterile inoculation loop and slowly immersed into the drop of hydrogen peroxide. Formation of bubbles was observed for positive test. In sugar fermentation test, to 5 ml of nutrient broth (containing 5µl phenol red), 100 µl of glucose (1%) solution was added. The test tubes were inoculated with bacterial culture and incubated at 37°C for 24-48 hrs. The change in colour of the medium from red to yellow was observed for the positive results of fermentation. In case of homo fermentation production of acid with the change in colour of the medium from red to yellow whereas in hetero fermentation gas production in durham tube are likely.

Results and Discussion

Twenty one bacteria were isolated from silkworm breeds, Double hybrid {(CSR 6 X CSR 26) X (CSR 2 X CSR 27)} from silkworm gut. Test isolates were studied for their morphological characteristics viz., type of colony, colour, surface, margin, elevation, opacity and presence of pigment and the results are presented in Table 1. Test isolates showed prominent growth on MRS agar after 24-48 hr of incubation. The colour of colonies ranged from off white, shiny white to creamy white. Margins were entire and undulate in all the isolates. Elevations of the isolated colonies were flat, raised and convex. The opacity of the isolates was translucent and opaque in nature. Further the colour pigments

were absent in all pure colonies of isolates and appeared white to creamish in colour. Test cultures of various cultural and biochemical tests studied for identification of lactic acid bacteria viz., motility test, oxidase test, endospore test, catalase and sugar fermentation test are summarized in Table 2. Grams staining of bacterial isolates were done for distinguishing between Gram +ve and Gram -ve bacteria.

The strains exhibited different cell shapes varying from rod to cocci. Out of 21 isolates 8 were Gram +ve rods whereas, 13 isolates were Gram +ve cocci in shape. Motility test showed that the isolates were non-motile, growing in the confined stab line. Endospore test revealed that bacteria were non-endospore forming. Catalase test study showed that in the entire isolates catalase enzyme was absent and they were not able to produce bubbles when mixed with H₂O₂. All the isolates were homo fermentative and glucose fermented to acid with change of the colour of medium from red to yellow.

Ten bacterial isolates were identified as Lactic acid bacteria group by morphological, cultural and biochemical characteristic study. The results from the present study were compared with the literature. Ahmed and Kanwal (2004) isolated 11 different strains of lactic acid bacteria from Camel milk and all the strains were non-motile. All the 21 isolates reported in the present study and isolated from different dairy samples were also non-motile. El- Hadi Sulieman *et al.*, (2006) reported that the majority of lactic acid bacteria isolated from Garris (Sudanese traditional fermented camel milk) belongs to the genus *Lactobacillus*. Forouhanden *et al.*, (2010) isolated 13 lactic acid forming bacteria from different traditional and local cheese and yoghurt. Biochemical characterizations of all the isolates were tested by the utilization of carbon sources.

Table.1 Morphological characteristics of cultures isolated on MRS agar from silkworm gut

S. No	Isolate name	Shape	Colour	Surface	Margin	Elevation	Pigment	Opacity
1	SWGL 1	Circular	Off white	Shiny	Entire	Convex	-	Opaque
2	SWGL 5	Circular	Creamy white	Shiny	Entire	Convex	-	Translucent
3	SWGL 6	Circular	Creamy white	Smooth	Entire	Raised	-	Translucent
4	SWGL 7	Circular	Creamy white	Rough	Entire	Raised	-	Translucent
5	SWGL 8	Circular	Off White	Shiny	Undulate	Convex	-	Opaque
6	SWGL 9	Circular	Creamy white	Rough	Undulate	Flat	-	Translucent
7	SWGL 10	Irregular	Creamy white	Smooth	Undulate	Raised	-	Opaque
8	SWGL 11	Circular	Shiny white	Smooth	Entire	Convex	-	Opaque
9	SWGL 13	Irregular	Creamy white	Shiny	Undulate	Convex	-	Translucent
10	SWGL 14	Circular	Creamy white	Shiny	Entire	Raised	-	Translucent
11	SWGL 15	Circular	Creamy white	Smooth	Entire	Convex	-	Opaque
12	SWGL 16	Circular	Creamy white	Shiny	Entire	Flat	-	Translucent
13	SWGL 17	Irregular	White	Shiny	Undulate	Convex	-	Translucent
14	SWGL 20	Circular	Creamy white	Shiny	Entire	Raised	-	Translucent
15	SWGL 21	Irregular	Creamy white	Shiny	Undulate	Flat	-	Opaque
16	SWGL 23	Circular	Creamy white	Smooth	Entire	Convex	-	Translucent
17	SWGL 30	Circular	Creamy white	Shiny	Entire	Convex	-	Translucent
18	SWGL 32	Irregular	Creamy white	Shiny	Undulate	Flat	-	Opaque
19	SWGL 32	Circular	Creamy white	Smooth	Entire	Convex	-	Opaque
20	SWGL 34	Circular	Creamy white	Shiny	Entire	Raised	-	Translucent
21	SWGL 40	Irregular	Creamy white	Shiny	Undulate	Raised	-	Opaque

Table.2 Culture and biochemical characteristics of pure isolates from silkworm gut samples

S. No	Isolate name	Cell Morphology	Gram staining	Spore formation	Catalase test	Oxidase test	Motility test	Sugar fermentation
1	SWGL 1	Rods	+	-	-	-	Non-motile	H
2	SWGL 5	Cocci	+	-	-	-	Non-motile	H
3	SWGL 6	Rods	+	-	-	-	Non-motile	H
4	SWGL 7	Rods	+	-	-	-	Non-motile	H
5	SWGL 8	Cocci	+	-	-	-	Non-motile	H
6	SWGL 9	Cocci	+	-	-	-	Non-motile	H
7	SWGL 10	Rods	+	-	-	-	Non-motile	H
8	SWGL 11	Cocci	+	-	-	-	Non-motile	H
9	SWGL 13	Rods	+	-	-	-	Non-motile	H
10	SWGL 14	Cocci	+	-	-	-	Non-motile	H
11	SWGL 15	Cocci	+	-	-	-	Non-motile	H
12	SWGL 16	Rods	+	-	-	-	Non-motile	H
13	SWGL 17	Cocci	+	-	-	-	Non-motile	H
14	SWGL 20	Cocci	+	-	-	-	Non-motile	H
15	SWGL 21	Rods	+	-	-	-	Non-motile	H
16	SWGL 23	Rods	+	-	-	-	Non-motile	H
17	SWGL 30	Rods	+	-	-	-	Non-motile	H
18	SWGL 32	Cocci	+	-	-	-	Non-motile	H
19	SWGL 32	Cocci	+	-	-	-	Non-motile	H
20	SWGL 34	Rods	+	-	-	-	Non-motile	H
21	SWGL 40	Cocci	+	-	-	-	Non-motile	H

+ = Activity present, - = Negative Test, H = Homo fermentation

During fermentation glucose to acid occurred with change in colour from red to yellow. In present study, all isolates showed change in colour of sugar from red to yellow during fermentation of glucose. Goyal *et al.*, (2012) isolated 28 different lactic acid forming bacterial strains from 14 curd samples. All the strains were gram positive, non-spore forming and all of them were catalase negative. Similar observations were reported in the present study.

In the present investigation, 21 bacterial isolates were isolated from silkworm gut. All the isolates were characterized on the basis of colony morphology and biochemical characteristics. Eight bacterial isolates (SWGL 6, SWGL 7, SWGL 8, SWGL 9, SWGL 10, SWGL 11, SWGL 23 and SWGL 30) were identified as lactic acid bacteria by morphological, cultural and biochemical characteristics. Further study of selected lactic acid bacteria isolates by 16S rDNA gene sequencing is required for its species identification.

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How to cite this article:

Saranya M., S. V. Krishnamoorthy, D. Balachandar and Tilak M. 2019. Isolation and Characterization of Indigenous Probiotic Bacteria from Silkworm (*Bombyx mori* L.) Gut. *Int.J.Curr.Microbiol.App.Sci*. 8(09): 2869-2874. doi: <https://doi.org/10.20546/ijcmas.2019.809.329>