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Isolation and Biochemical Characterization of Lactic Acid Bacteria from the Gut of Fresh Food Fishes

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Abstract: This study aimed to propose the characterization of lactic acid bacteria (LAB) from the gut of common five marine fishes using morphological, physiological and biochemical characteristics. Fishes *Sillago sihama, Rastrelliger kanagurta, Mugil cephalus, Sardinella longiceps* and *Nemipterus japonicus* were collected from Kaasimedu fish market, Chennai, India. Lactic acid producing bacteria were isolated by using MRS broth. From inoculum, the bacterial colonies were observed in MRS agar using streak plate technique and incubated. Different colonies were observed and the colonies were sub-cultured for further study. Five different types of pure cultures were obtained from *Sillago sihama, Rastrelliger kanagurta, Mugil cephalus, Sardinella longiceps* and *Nemipterus japonicus* in MRS agar plates and named as LAB 1, LAB 2, LAB 3, LAB 4 and LAB 5, respectively and subjected for gram staining and catalase test to confirm the Lactic acid bacteria by biochemical method. The different small white colonies with smooth, rounded, entire margins were observed LAB 1, LAB 2, LAB 3, LAB 4 and LAB 5 in *Sillago sihama, Rastrelliger kanagurta, Mugil cephalus, Sardinella longiceps* and *Nemipterus japonicus,* respectively. The antibiotics and susceptibility test for LAB 2 and LAB 5 showed reasonable activity against *Escherichia coli* and *Staphylococcus aureus.* LAB 1 was sensitive to gentamycin, erythromycin and ampicillin drugs. These antibiotics are therefore harmful and destroy this strain of gut flora. However, LAB 1 was resistant to norflaxacin, which is a common antibiotic used to treat stomach disorders like dysentery. This confirmed the action of norfloxacin drug, which restoreed the internal environment of the stomach without destroying gut flora. LAB 5 showed intermediate sensitivity to gentamycin and ampicillin. LAB 4 was also resistant to norfloxacin. It is suggested that the lactic acid bacteria isolated from the fish gut has probiotic properties, especially with reference to being able to inhibit pathogenic strains. Nevertheless, lactic acid bacteria have several beneficial properties and develop novel functional properties for inhibition of microbes by producing growth inhibiting substances and large amounts of lactic acid.
Keywords: Lactic acid bacteria, Marine fishes, Gut microflora, Antibiotic test

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Introduction

The typical lactic acid bacterium is gram positive, non-sporulating, catalase negative and devoid of cytochromes. Lactic acid bacteria (LAB) are of non-aerobic habitat but aero-tolerant, fastidious, acid tolerant and strictly fermentative, with lactic acid as the major end product during sugar fermentation. Lactic acid bacteria are generally associated with habitats rich in nutrients, such as various food products (milk, meat, beverages, and vegetables), but some are also members of the normal flora of the mouth and intestine. This makes LAB devoid of a "true" catalase and cytochromes when grown in laboratory growth media, which lack heme or related compounds. Microflora of the intestinal tract is an integral part of the whole living organism. A great number of endogenous and exogenous factors determining the number and species composition of microbe populations and affecting physiological and biochemical features influence it. Many different bacteria get into an organism from the environment. However, due to natural selection, only those bacteria survive which find favorable living conditions in that organism (Khalil *et al.*, 2009). The most characteristic microbes associated with the digestive tract are "normal gut flora." These microorganisms in the digestive tract play important roles in nutrient metabolism, restriction of pathogenic microorganisms, maintaining animal health and improving production. In healthy animals, the composition of gut microflora remains in a relatively healthy state. If the composition of microflora is out of balance, induced by either internal factors (eg., allergic reactions) or external factors (e.g., food, environment, stress, antibiotic administration), pathogenic organisms may colonize the gut and lead to diarrhea, digestive disorders, poor production and death (Qingqiang and Qiuhong, 2004).

Lactic acid bacteria are widely distributed in various animal intestines (Devriese *et al.*, 1987). These lactic acid bacteria are the most prominent members of the gut microflora in animals. Many lactic acid bacteria are proved to function as probiotics, which are beneficial to host health, when ingested in sufficient quantities. The colonization of the gut by probiotic bacteria prevents growth of harmful bacteria by competition, exclusion and by the production of organic acid and antimicrobial compounds (Nirunya *et al.*, 2007). The acid and bile tolerance are two fundamental properties that indicate the ability of the probiotic organism to survive the passage through the upper gastrointestinal tract, particularly the acidic condition of the stomach and the presence of bile in the small intestine (Hyronimus *et al.*, 2000). Promising probiotic strains include members of the genera *Lactobacillus, Enterococcus* and *Bifidobacterium.* The representative species include *Lactobacillus acidophilus, Lactobacillus johnsonii, Lactobacillus casei, Lactobacillus gasseri, Lactobacillus plantarum, Lactobacillus rhamnosus, Bifidobacterium longum, Bifidobacterium breve, Bifidobacterium bifidum, Bifidobacterium infantis, Enterococcus faecalis* and *Enterococccus faecium* (Kaur *et al.*, 2002).

The ability of lactic acid bacteria to inhibit the growth of undesired bacteria is well known. This inhibition may be due to numerous factors such as the production of organic acids and hydrogen peroxide, CO_2, nutrient depletion, decrease in redox potential and synthesis of antibiotics and bacteriocins (Klaenhammer, 1993). The production of organic acids (lactic, acetic and propionic acids) has an antagonistic effect on the microbiota by the inhibition of active transport processes, reactions and the modifications of their membrane potential. Among them, acetic acid has the most severe antimicrobial effect and it acts over fungi, yeast and bacteria (Blom and Morvedt, 1991). LAB's lack of catalase causes an accumulation of hydrogen peroxide (H_2O_2), which is able to inhibit bacteria and fungi (Caplice and Fitzgerald, 1999). The carbon dioxide produced during heterolactic fermentations create an anaerobic environment which is toxic for some aerobic bacteria, it also contributes to decrease pH. A few LAB strains are able to produce protein

compounds with a noteworthy antimicrobial effect, which are known as bacteriocins (Ross *et al.*, 2002).

Bacteriocins are antimicrobial proteinaceous compounds that are inhibitory towards sensitive strains and are produced by both Gram-positive and Gram-negative bacteria (Tagg and McGiven, 1976). Bacteriocins are commonly divided into groups. Class I, termed as lantibiotics, mainly represented by nisin (Breukink and Kruijff, 1999). This class is formed by peptides of 19 to more than 50 amino acids which are characterized by their unusual amino acids such as lanthionine, methyl-lanthionine, dehydorbutyrine and dehydroalanine. Class I is subdivided into subclasses Ia and Ib. Class Ia bacteriocins, i.e. nisin, consists of cationic and hydrophobic peptides that form pores in target membranes. Class Ib bateriocins are globular peptides with no net charge or a net negative charge with a more rigid structure compared with class Ia. Class II comprises heat stable, non-modified peptides and can further be subdivided. Class II a includes Pediocin -- like *Listeria* active peptides with a conserved N-terminal sequence Tyr–Gly- Asn-Gly-Val and two cysteines forming a S-S bridge in the N-terminal half of the peptide (Eijsink *et al.*, 1998). The two peptide bacteriocins, such as lactococcin and lactacin, form the class IIb.

Thus we understand the important role that lactic acid bacteria play in maintaining healthy conditions in a wide group of animals. Their role is not restricted to fish alone, but can be extended to human beings, as well as poultry. Since, majority of lactic acid bacteria are 'beneficial bacteria', they serve as easily available raw material for the synthesis of several probiotic administrations. It is this unique probiotic ability of lactic acid bacteria which provides a broad platform for several advances in medical research. This study aims at establishing the role of lactic acid bacteria in the marine environment. The main purpose of this work was to point out the easy availability of lactic acid bacteria from common marine fish. Five species were chosen for this study – *Sillago sihama, Rastrelliger kanagurta, Mugil cephalus, Sardinella longiceps* and *Nemipterus japonicus.* Successful isolation of LAB from fresh marine fish, gave way for further morphological, biochemical and physiological study. Thus, through this work the role of LAB in maintaining fish health and the scope it provides for improving the health of other animals, including man could be clearly understood.

Materials and Methods

Five species of fresh fish (marine) were procured from Kaasimedu fish market. They are sand whiting (*Sillago sihama*) (Fig. 1), mackerel (*Rastrelliger kanagurta*) (Fig. 2), grey mullet (*Mugil cephalus* (Fig. 3), oil sardine (*Sardinella longiceps*) (Fig. 4) and thread fin bream (*Nemipterus japonicus*) (Fig. 5). The fishes were transported in iceboxes to the lab, where they were dissected aseptically in order to remove the intestine. One gram of intestine content was homogenized with 9 ml of sterile saline and vortexed for one minute in a stomacher. 0.5 ml of this well blended content was then transferred into sterile MRS broth tubes. The tubes were incubated anaerobically for 24 h at 37 C. Turbidity in the tubes indicated growth. Then, inoculum from these tubes was streak plated onto MRS agar plates, which were incubated anaerobically for 24 h at 37 C. Well isolated colonies with typical characteristics, namely pure white, small (2-3 mm), with entire margins were picked up and sub-cultured thrice for purification and confirmation, before proceeding with the experiment.

Fig. 1: *Sillago sihama.*

Fig.2: *Rastrelliger kanagurta.*

Fig. 3: *Mugil cephalus.*

Fig. 4: *Sardinella longiceps.*

Fig. 5: *Nemipterus japonicus.*

Five sets of pure cultures were obtained in all and were maintained aseptically on MRS agar plates. They were labeled as LAB 1 (sand whiting), LAB 2 (mackerel), LAB 3 (mullet), LAB 4 (sardine) and LAB 5 (thread fin bream).

Results and Discussion

Bacteriological analysis:

The bacterial cultures were subjected to the confirmatory tests (Gram staining and Catalase test), which clearly indicated that the isolated bacteria are Lactic acid bacteria (LAB). Subsequently, these five cultures were then distinguished based on their biochemical characteristics. This was enabled through the following tests like (i) Indole production test, (ii) Methyl Red test, (iii) Voges-Proskaeur, and (iv) Citrate utilization and Carbohydrate fermentation of D-glucose, sucrose, lactose, D-xylose, mannitol, mannose, maltose and arabinose. The homogenized intestinal contents of the different fish were plated on MRS agar and incubated anaerobically. Typical lactic acid bacteria colonies were picked up and purified thrice, before they were finally used for the experiment. The distinguishing characteristics of lactic acid bacteria were typical small white colonies with smooth, rounded, entire margins (Figs. 6-10). Five separate cultures were obtained from *Sillago sihama, Rastrelliger kanagurta, Mugil cephalus, Sardinella longiceps and Nempiterus japonicus* and were designated as LAB 1, LAB 2, LAB 3, LAB 4 and LAB 5, respectively.

For each of the above cultures, three confirmatory tests were performed: Gram staining, catalase test and spore formation. All bacteria were found to be Gram-positive, catalase-negative and non-sporulating (Table 1). The biochemical analysis of the five lactic acid bacteria cultures was performed. All species of the lactic acid bacteria isolated from *Sillago sihama, Rastrelliger kanagurta, Mugil cephalus, Sardinella longiceps and Nemipterus japonicus* were found to be indole negative (Fig. 11). This implies that the bacteria are incapable of breaking down tryptophan to yield indole. For the methyl red test, all the five cultures tested positive, indicated by the formation of a red color (Fig. 12). This positive result indicated that glucose present in MRVP broth has been broken down by lactic acid bacteria into acidic end products. Formation of

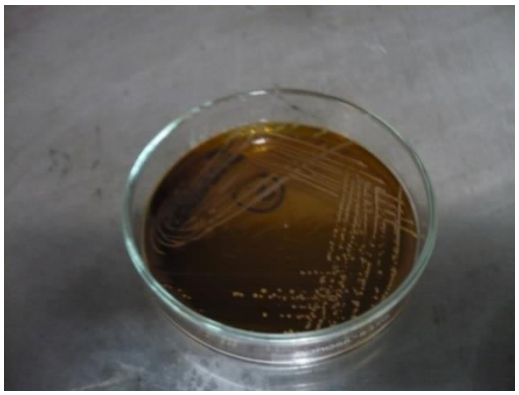

Fig. 6: Lactic Acid Bacteria from *Rastrelliger kanagurta.*

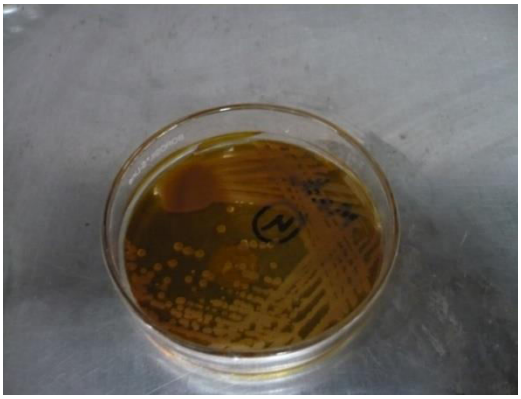

Fig. 7: Lactic Acid Bacteria from *Sillago sihama.*

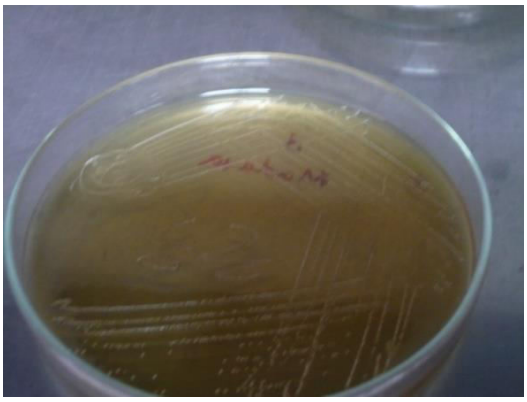

Fig. 8: Lactic Acid Bacteria from *Mugil cephalus.*

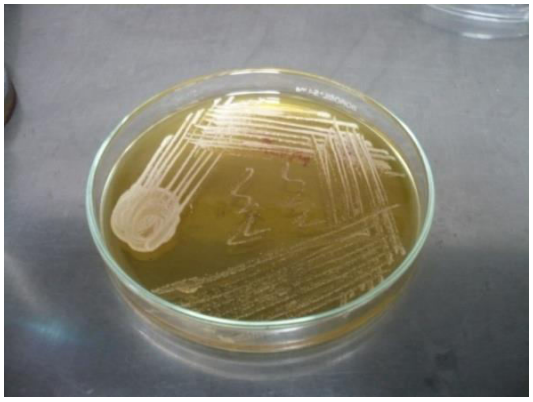

Fig. 9: Lactic Acid Bacteria from *Sardinella longiceps.*

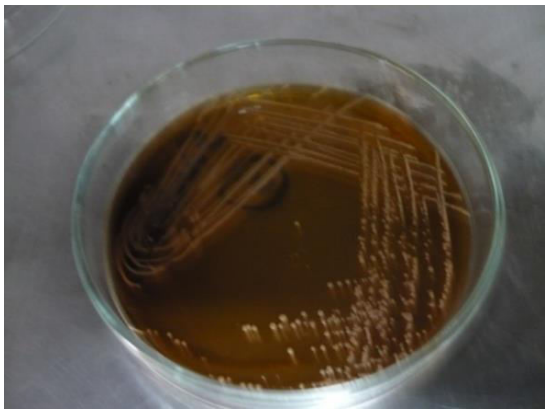

Fig. 10: Lactic Acid Bacteria from *Nemipterus japonicus.*

acidic end products is a characteristic feature of LAB. LAB 1-5 tested negative for the Voges-Proskaeur test (Fig. 13). These negative results confirmed the act that glucose present in MRVP medium has not been converted to acetoin by lactic acid bacteria. LAB 4 (*Sardinella longiceps)* and LAB 5 (*Nemipterus japonicus)* showed positive results for the Citrate Utilization tests, by the development of a deep blue color (Fig. 14). From this change in color, it is understood that lactic acid bacteria isolated from *Sardinella longiceps* and *Nemipterus japonicus* have the ability to utilize citrate present in the medium, as the sole carbon source for growth (Table 2).

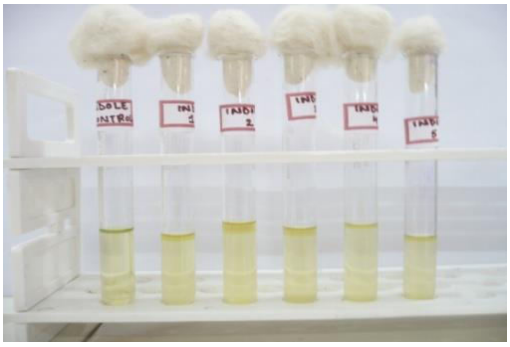

Fig. 11: Indole Negative.

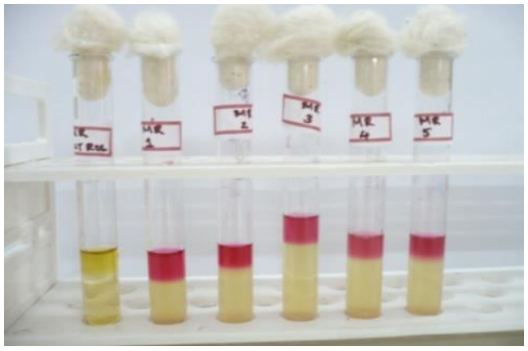

Fig.12: Methyl Red positive.

Fig.13: Voges-Proskaeur negative.

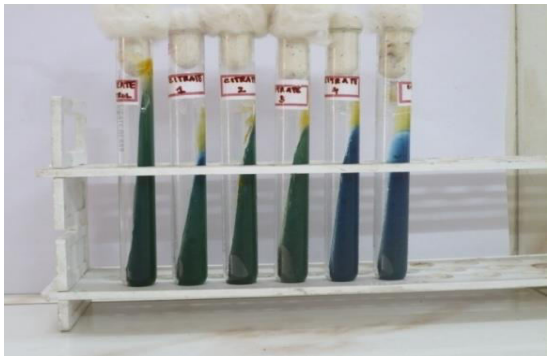

Fig.14: Citrate Utilization.

Table 1: Confirmatory tests for lactic acid bacteria

TESTS	LAB 1	LAB 2	LAB 3	LAB 4	LAB 5
Gram Staining	+	+	+	+	+
Catalase test	-	-	-	-	-
Spore formation	-	-	-	-	-

Table 2: Biochemical analysis of lactic acid bacteria

TESTS	LAB 1	LAB 2	LAB 3	LAB 4	LAB 5
Indole test	-	-	-	-	-
Methyl Red test	+	+	+	+	+
VogesProskaeur test	-	-	-	-	-
Citrate utilization test	-	-	-	+	+

LAB 1-5 exhibited clear distinguishing sugar fermentation properties. LAB 1 (Fig. 15) was unable to ferment xylose and mannitol, whereas it showed acid and gas production from mannose. LAB 2 (Fig. 16) showed a 50% fermentation of the eight sugars chosen. Acid production was seen from glucose, sucrose, xylose and maltose. LAB 3 (Fig. 17) was able to produce acid from glucose, sucrose, lactose and maltose. LAB 4 was unable to ferment only xylose and arabinose. LAB 4 (Fig. 18)

showed acid and gas production from mannose. LAB 5 (Fig. 19) was unable to ferment only mannose. All the other 7 sugars were fermented by LAB 5 (Table 3).

Fig. 15: LAB 1.

Fig. 16: LAB 2.

Fig. 17: LAB 3.

Fig. 18: LAB 4.

Fig. 19: LAB 5.

Table 3: Differential sugar fermenting characteristics

SUGAR	LAB 1	LAB 2	LAB 3	LAB 4	LAB 5
Glucose	+	+	+	+	+
Sucrose	+	+	+	+	+
Lactose	+	-	+	+	+
Xylose	-	+	-	-	+
Maltose	+	+	+	+	+
Mannitol	-	-	-	+	+
Mannose	+ (gas)	-	-	+ (gas)	-
Arabinose	+	-	-	-	+

Antibiotic activity and susceptibility:

LAB 2 and LAB 5 showed reasonable activity against *Escherichia coli* and *Staphylococcus aureus.* LAB 1 is sensitive to gentamycin, erythromycin and ampicillin drugs (Table 4). These antibiotics are therefore harmful and destroy this strain of gut flora. However, LAB 1 is resistant to norflaxacin, which is a common antibiotic used to treat stomach disorders like dysentery. This confirms the action of norfloxacin drug, which restores the internal environment of the stomach without destroying gut flora (Fig. 20). LAB 5 shows intermediate sensitivity to gentamycin and ampicillin. LAB 4 is also resistant to norfloxacin (Fig. 21) (Results have been interpreted based on 'Zone size Interpretative chart'–DA approved performance standards. Source: HiMedia Laboratories Pvt. Ltd., Mumbai).

The rich diversity of microorganisms in the gut of all animals is a well-established fact. This study has, in particular dealt with lactic acid bacteria associated with the fish gut. Fresh marine fish from the nearest landing centre (Kaasimedu) were chosen for study – *Silago sihama, Rastrelliger*

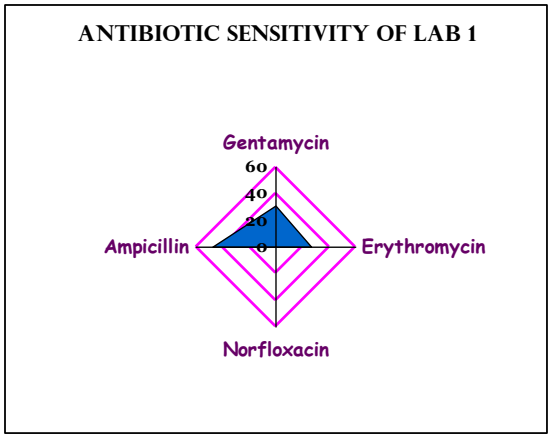

Fig. 20: Graphical representation of antibiotic sensitivity (LAB 1).

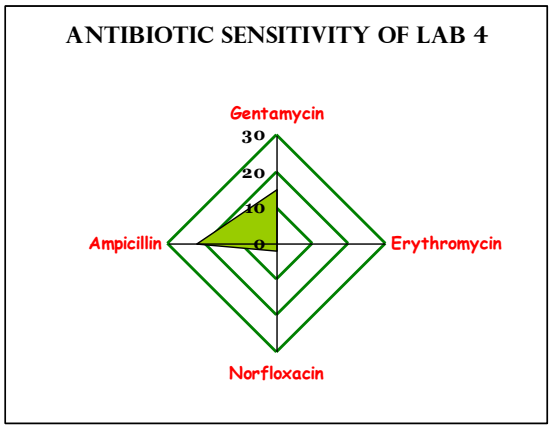

Fig. 21: Graphical representation of antibiotic sensitivity (LAB 4).

Table 4: Antibiotic susceptibility

ANTIBIOTIC	LAB 1	LAB 4
Gentamycin	31 mm	15 mm
Erythromycin	26 mm	-
Norfloxacin	0 mm	2 mm
Ampicillin	47 mm	22 mm

kanagurta, Mugil cephalus, Sardinella longiceps and *Nemipterus japonicus.* LAB was successfully isolated from the gut of healthy, fresh marine fish, using MRS agar (Sugita *et al.*, 1985; Ringo and Gatesoupe, 1998). The isolation of LAB was confirmed using some standard tests. All the isolated strains were Gram-positive and non-sporulating, which is in agreement with previously done work (Nair and Surendran, 2004). Results of the biochemical tests done also showed similarity to prior work (Ahmed and Kanwal, 2004). All five LAB cultures were methyl red positive which is in conformity with the work of Jack *et al.* (1995).

The antimicrobial properties of lactic acid bacteria has already been studied in case of several other fish and was found to have yielded positive results. Lactic acid bacteria owe their antimicrobial property to the production of bacteriocins (Geesje *et al.,* 1992). The present study also indicated the anti-pathogenic effects of lactic acid bacteria against *Escherichia coli* and *Staphylococcus aureus.* The zone of inhibition formed confirmed the antibacterial activity of the isolated LAB against the selected pathogens (Rammselberg and Radler, 1990). The inhibitory effects of *Bacillus* sp. may be due to production of antibiotics, bacteriocins, lysozymes, proteases and/or hydrogen peroxide and the alteration of pH values by the production of organic acids. Thus from results of this study it can be suggested that lactic acid bacteria isolated from the fish gut has probiotic properties, especially with reference to being able to inhibit pathogenic strains. This ability of LAB enhances its importance in the medical field. Lactic acid bacteria isolated from fish gut can be converted into suitable form and administered to reestablish healthy intestinal conditions and maintain normal gut flora.

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