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GUT-ISOLATED BACTERIA AND BIOCHEMICAL COMPOSITION ISOLATED FROM EDIBLE CRAB SPECIES IN MANAPAD, INDIA

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ABSTRACT

Crabs constitute an important resource in the marine fishery in India and contributed an overall average of 9.6% to the total crustacean landings from 1975-to 2020. Many species of crabs are exploited alonIndia's east and west coasts, mainly in trawls as by-catch and as a targeted resource in a gill net in some regions. In Tamil Nadu, the hitherto dominating species *Portunus pelagicus* (30.7%) has been overtaken by *Portunus sanguinolentus* (33.3%) with a marginal increase registered in the landings. Crabs proteins are rich in essential amino acids (EAA). Muscle proteins are grouped into three categories based on location in the skeletal muscle and solubility sarcoplasmic, stromal, and myofibrillar proteins. The survival, health, growth, and efficient feed utilization of fish depend on the nutritional value of feedstuff. Fish, like any other animal, do not have a nutritional requirement of protein *per se* but require a well-balanced mixture of indispensable or essential amino acids (EAA) and dispensable or nonessential amino acids. Using the 16S ribosomal RNA sequences (Bacteria) database and the sequences of purified PCR products from the final 2 isolates (ON248499 and ON248500), the National Center for Biotechnology Information's (NCBI) BLAST programme. The results of the current analysis showed that *P.sanguinolentus* tissue has the most protein compared to other tissues. The findings of those who claimed that *P.pelagicus* had has a decent amount of protein match well with those of the current investigation, the

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level of total lipid in the body tissues of crab *Portunus pelagicus* and *Portunus sanguinolentus* is (0.3% and 2.3%) As a result, the current study aimed to increase seafood intake among the general public by emphasising the nutritional importance of seafood, particularly crabs, in the local community.

KEYWORDS: Blue swimmer crab, three spotted crab, NCBI, Biomolecules and nutritional factors

INTRODUCTION

Crabs constitute an important resource in the marine fishery in India and contributed an overall average of 9.6% to the total crustacean landings from 1975-to 2020. Many species of crabs are exploited along the east and west coasts of India, mainly in trawls as by-catch and as a targeted resource in a gill net in some regions. Edible crabs landed in India belong to the family Portunidae and around 61% of the landings were recorded by three species of marine crabs three spotted crab (*Portunus sanguinolentus)* (*Herbst*, 1783 *)* (28.2%), blue swimmer crab (*Portunus pelagicus*) (Linné Carl von & Salvius Lars, 1758) (25%) and Charybdis feriata (7.7%). In Tamil Nadu, the hitherto dominating species *Portunus pelagicus* (30.7%) has been overtaken by *Portunus sanguinolentus* (33.3%) with a marginal increase registered in the landings. (Josileen et al., 2021). The crab is considered a vital member of decapod crustaceans belonging to the shellfish group and living in marine, brackish, or freshwaters habitat. Nowadays it's become a popular fisheries item and a specific seafood product among other seafood in India. (Wan Yusof et al., 2019). Among shellfish products, crab meat is the most demanded seafood around the globe due to its nutritional value (Sarower et al., 2013). Crabmeat is reported as providing nourishment to the human body thus increasing cognition, reducing inflammation, strengthening the bones, boosting the immune system, stimulating blood circulation and detoxifying the body (Who, 2010). These are usually found in large numbers in shallow bays with sandy bottoms at about 50-60m depth (Edgar, 1990). Seafood is easily digestible because it has very little connective tissue. For that reason, fish is recommended in many special diets. (Ravichandran S, 2013). Gut microbes play important roles in the physiological processes of the host. Gut bacterial community composition depends on the host's developmental stage and is strongly influenced by various interacting host-associated factors that affect its growth and development, both inside and outside the gut (Angthong et al., 2020). The amylase-producing ability of intestinal bacteria in crab and 7 fish species has been determined Bacillus, Coryneforms, Enterobacteriaceae, Flavobacterium, Micrococcus, Pseudomonas and Vibrio species were found in the intestine tracts of Japanese flounder (Sugita et al., 1996). Internal microbiota can play a role in nutrition, immunity, and disease resistance of animals. The relationship between environmental factors and the microbial community structure in the water of crab culture ponds was also investigated (Hou et al., 2017). Some researchers analyzed differences in the intestinal bacteria between pond-raised and wild crabs(Li et al., 2007) and the microbial diversity in the sediment of a crab pond (Liu et al., 2013). The composition and diversity of bacterial communities in the crab

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intestine, water, and sediment in rice-crab co-culture and crab monoculture models have been characterized (X. Chen et al., 2015)

Crabs proteins are rich in essential amino acids (EAA). Muscle proteins are grouped into three categories based on location in the skeletal muscle and solubility sarcoplasmic, stromal, and myofibrillar proteins. Myofibrillar proteins are the main component of the skeletal muscle accounting for about 50% of total proteins and are mainly constituted by myosin and actin, involved in muscle contraction. Lipids are major sources of metabolic energy and essential materials for the formation of cell and tissue membranes. They are very important in the physiology and reproductive process of marine animals and reflect the special biochemical and ecological conditions of the marine environment (Sargent, 1995). As one of the three major nutrients, carbohydrates are preferentially broken down when supplying energy to the body(Council, 2011). Providing adequate amounts of digestible carbohydrates in diets formulated to decrease ammonia excretion and water pollution(BeMiller & Huber, 2008). The survival, health, growth, and efficient feed utilization of fish depend on the nutritional value of feedstuff. Fish, like any other animal, do not have a nutritional requirement of protein *per se* but require a well-balanced mixture of indispensable or essential amino acids (EAA) and dispensable or nonessential amino acids. Usually, dose-response trials are used to determine amino acid requirements in fish. This is a costly and time-consuming method, especially when aimed at determining requirements for all EAA (Akiyama et al. 1997). There are around a lot of crab species found along the Indian coastal waters which found that crab is a good source of nutrition and valuable fishery product, commercially important edible crabs are known as *Portunus sanguinolentus* and *Portunus pelagicus* having good potential export as well as local consumption. The main goal of this study was to evaluate the biochemical features of two different edible commercial crab species obtained from India's coastal area to provide baseline data on nutritional components. As a result, the current study aimed to increase seafood intake among the general public by emphasising the nutritional importance of seafood, particularly crabs, in the local community.

MATERIAL AND METHODS

Crabs of 12+1 cm in length were selected for this study. They were cleaned in chlorinated water, and the length and breadth of their carapaces were measured and the animal's total weight. The weight of the orange-red coloured eggs in the intestinal cavity was determined when the carapace was removed. The surfaces of the crabs were sterilized with 70% ethanol. The intestines were aseptically dissected from the musculature and placed into a 2-mL sterile centrifuge tube. The crab body was cleansed of gills and intestines, and the flesh was picked up and weighed by cutting off the intermittent shells. The claw shell was cut open with scissors, the liquor was kept, and weights were calculated, as well as the weight of the clawed flesh. The muscle was minced after cooling to 0º C. This minced muscle was collected for a variety of tests.

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- **DNA EXTRACTION AND SEQUENCING**

i) Sample preparation

The gut tissue of the collected individuals was extracted immediately under natural light. 1ml was transferred aseptically to a 9ml blank and mixed thoroughly. Similarly, serial dilutions were made and used as inocula. For gut analysis, the digestive system was dissected out aseptically using sterile scissors and forceps. Gut was homogenized in a tissue homogenizer and transferred to a 99ml blank, from the suspensions, 1ml of the sample was pipetted out and added 9ml blank; likewise, serial dilutions were made. For all the samples triplicates were inoculated at a temperature of 28±1ºC for 2-7 days and the colonies were counted. The microbial load was counted and expressed as the number of colony-forming units (CFU). The bacteria were identified to a generic level.

ii) Genomic DNA isolation from bacteria

A part of the culture is taken in a microcentrifuge tube. 180µl of T1 buffer and 25µl of proteinase weweredded and incubated at 56 º C in a water bath until it was completely lysed. After lysis, 5 µl of RNase A (100 mg/ml) was added and incubated at room temperature for 5 minutes. 200 µl of 100% ethanol was added and mixed thoroughly by vortexing. The mixture was pipetted into a NucleoSpin® Tissue column placed in a 2 ml collection tube and centrifuged at 11000 × g for 1 minute. The NucleoSpin® Tissue column was transferred to a new 2ml tube and washed with 500 µl of BW buffer. The Wash step was repeated using 600 µl of B5 buffer. After washing the NucleoSpin® Tissue column was placed in a clean 1.5ml tube and DNA was eluted out using 50 µl of BE buffer.

iii) PCR analysis

We used PCR to amplify the bacterial 16S rRNA gene. The primers used in the present study were 338F (5′-barcode-CAGGCCTAACACATGCAAGTC3′) and 806R (5′-GGGCGGWGTGTACAAGGC-3′) (amplicon length: 468 bp), 20 µL of PCR mixture containing 5×D/W (6.6 µL), 5X buffer (1.9 µL), forward primer (0.3 µL), reverse primer (0.3 µL), sequencing mixer (0.2 µL), Exosap treated PCR product (1 µL). The cleaned-up air-dried product was sequenced in ABI 3500 DNA Analyzer (Applied Biosystems) using the Sanger DNA sequencing method.

iv) Sequence analysis

The sequence quality was checked using Sequence Scanner Software v1 (Applied Biosystems). Sequence alignment and required editing of the obtained the sequences were carried out using Geneious Pro v5.1 (Drummond et al., 2010)

- **Determination of Protein**

The flowchart of the extraction of muscle protein fractions from different species analyzed is shown in Figure 1. Meat and fish proteins were fractionated based on different solubility.

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Samples were homogenized with 0.03 M phosphate buffer (pH 7) containing a protease. All extracted samples were centrifuged for 15 min at 3,000 rpm, filtered and made to the standard volume. One ml extract of each fraction containing (3~6 mg protein), in duplicate, was taken for estimation by biuret method, with a Double Beam Spectrophotometer at 540nm. Samples were treated with petroleum ether to remove any lipid contamination before developing the colour. Bovine albumin was used to prepare the standard curves for low ionic, and high ionic strength and 0, 1 M NaOH extracts. The standard curves were made after applying the appropriate correction. The acid-soluble fraction of stroma, however, was determined by the method of Lowry et al., because the biuret method, despite its certain advantages over the others, is not sensitive enough to measure the protein in a very low concentration.

Taken 500 mg of dry tissue (body muscle)

▯

Added 6 ml of saline

▯

Centrifuged for 15 - 20 min for 4000 rpm

▯

1 ml of supernatant and added 5 ml of Reagent (C)

▯

Incubated for 10 min.

▯

Added 0.5 ml of Reagent (D)

▯

Incubated for 30 min.

▯

Measured the O. D. at 540 nm

- **Determination of carbohydrates**

For the estimation of total carbohydrate content, the procedure of using phenol - Sulphuric acid was followed. About 5 mg of the oven-dried tissue was taken for carbohydrate analysis. The tissue was taken in a test tube and 1 ml of Phenol (5%) and 5ml concentrate H2SO4 were added in quick succession. The tube was kept for 30 min at 30°C and the optical density (OD) of the coloured developed was measured at 490nm against the blank. D-Glucose was used as a standard and it had an optical density value of 0.1 Carbohydrate as calculated by using the formula.

Percentage (%) of Carbohydrate = Standard value × OD of sample / Weight ×100

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- **Analysis of Fatty Acids**

Samples for fatty acid composition in the muscle and subcutaneous tissue (between the 12th and 13th ribs) were collected, vacuum-packed, and stored at -20°C until analysis The quantification of fatty acids in the meat samples was done according to the method described as follows. Total lipid from muscle and subcutaneous tissue samples were extracted according to the method of Folch et al. using chloroform and methanol in a ratio of 2:1. Briefly, 10 g of sample was mixed with 200 mL of chloroform: methanol (2: 1, v/v). Then the mixture was vortexed, centrifuged, and filtered. After that, the 0.9% NaCl solution was addedto the solvent and centrifuged again to form the layers. The upper layer of the solvent was removed by siphoning and the lower layer (chloroform containing total lipids) was collected. From the collected layer, the chloroform was removed under vacuum at 40°C and extracted fat was collected and content was determined gravimetrically. The percentage of Crude lipid content was calculated by the following equation.

Percentage (%) of fat = (Weight of extract/Weight of sample) × 100

- **Determination of moisture**

Moisture was determined by keeping the weighed quantity of tissue in a thermostat-controlled oven at 105˚ C for 6 hours. Thereafter samples were kept in a desiccator for about 1 hour. The dry weight of each sample was taken in an electric balance. The percentage of the moisture content was then calculated by the following formula:

Percentage (%) of moisture = (Weight of loss / original weight of the sample) × 100

- **Determination of ash**

Ash content was determined by igniting a previously dried tissue sample in a muffle furnace at 550º C for 6 hours. The ash content was calculated by the following equation.

Percentage (%) of ash = (Weight of ash / Weight of sample) × 100

RESULT

- **IDENTIFICATION OF BACTERIAL ISOLATES**

Using the 16S ribosomal RNA sequences (Bacteria) database and the sequences of purified PCR products from the final 2 isolates, the National Center for Biotechnology Information's (NCBI) BLAST programme (Figures 1 & 2) was used to identify the isolates. The isolates MB1 and MB2 partial sequences were utilised. Genus and species identifications were made for sequences with a similarity rate of 96% or above. Genus alone was used to identify sequences with 93–96% similarity. The metagenomic sequences were deposited at the NCBI under accession numbers ON248499 and ON248500 (Table 1) The inserts ranged from 490 to 527 bp in size. Based on the RDP classifier and phylogenetic analysis, the cloned sequences

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- **BIOCHEMICAL ANALYSIS**

The protein composition of crab from the Manapad Estuary was quantitatively analysed. The findings demonstrated that both of the crabs that were gathered from the location had significant protein levels. Quantitative analysis of protein content of two different species of crab was carried out based on the method of Lowry et.al. *P.pelagicus* muscle tissue containing (23.6%) protein. Additionally, *P. sanguinolentus* has (25.3%) protein. The results showed that among the two different species identified *Portunus sanguinolentus* had the highest protein content (25.3%). Quantitative analysis of carbohydrate content of two samples showed that *P.sanguinolentus* had (1.3%) carbohydrate and *P.pelagicus had* (3.6%) carbohydrate. Moisture content was maximum in *Portunus pelagicus* (60.31%) and *Portunus sanguinolentus* (55.06%). Ash content was maximum in *Portunus pelagicus* (11.40%) and *Portunus sanguinolentus* (10.52%). (Table 2)

DISCUSSION

India's crab fishery is rapidly expanding, and because crab flesh is so delectable and nutrient-dense, there is a huge market for it. Crab meat has recently gained popularity on a worldwide scale, and is now widely fished for and sold in all of India's maritime nations as well as internationally. (Mk & Suseelan, 2001) The circadian clock, which regulates the host's metabolism, is tightly tied to the dynamics of the gut microbiome. (Murakami et al., 2016). There is growing proof that the immune system and daily rhythm are closely related, showing that immune activity varies over the 24-hour day-night cycle (S.-T. Chen et al., 1989). The environment, nutrition, genetics, and other factors all have an impact on the microbiota of the digestive system. Early studies suggested that gut microorganisms play a crucial role in the absorption and breakdown of nutrients (Zhang et al., 2014). There is mounting evidence linking ambient microorganisms to crab digestive system illnesses caused by bacteria. Recently, some studies have concentrated on the bacterial community composition in cultured crab water (Cheng et al., 2010), sediment (Liu et al., 2013), and crab intestines (Li et al., 2007). Other studies have also concentrated on a comparison of the microbiota from the intestine of E. sinensis in rice-crab co. Different writers reported on the protein content of different crabs. P. pelagicus and P. sanguinolentus had protein contents of 0.47 to 15.91 per cent and 12.81 to 13.6 per cent, respectively. The protein serves a variety of purposes and offers 5.65 Kcal of energy per g. The RDA for protein is one gramme per kilogramme of body weight (adults). The need for protein changes with age, physiological condition, and stress. Children who are growing, pregnant women, nursing mothers, and people who are unwell or under stress all use more proteins. (Ravichandran S, 2013) The results of the current analysis showed that *P.sanguinolentus* tissue has the most protein compared to other tissues. The findings of those who claimed that *P.pelagicus* has a decent amount of protein match well with those of the current investigation. Lipids play a crucial role in preserving the physiological and structural integrity of cellular and sub-cellular membranes. The best source of energy for the body to create through metabolism is lipids. In the present study, the level of total lipid in the body tissues of crab *Portunus pelagicus* and *Portunus sanguinolentus* is

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(0.3% and 2.3%). The results of the present study are consistent with those of *Callinectes amnicola* (Kathirvel et al., 2014) on *Calappalophus* (Srilatha et al., 2013) on the crab *Emerita emeritus*. Any species' variation in moisture content is dependent on the types of food they ate (Soundarapandian & Murugesan, 2010)(El-Gendy et al., 2018) factors such as flavour, texture, and The moisture content has a significant act on and influence the body weight. (S & V, 2020)Vigneshwari and Gokula Similar to how seasonal changes in prior investigations also noted the presence of moisture, it relies on the dietary status, physiological state, and reproductive cycle of a creature (Singh et al., 2013). Moisture is a major constituent in animal body which plays a vital role in regulating osmotic functions. It serves as a medium through which nutrients are transported to different organs. Water is so vital that an animal can practically lose all of its fat and half of its protein and still live, but loss of even 10% of its water can cause death.(Loosli et al., 2022)

The current study showed that aquatic animals, particularly crab, are often consumed by people in the nation and are a solid source of affordable animal protein, both as snacks and as a component of main meals.The profile of carbs, proteins, lipids, amino acids, and fatty acids displayed the highest values in the current study. It suggests that the primary suppliers of animal proteins are crabs. Therefore, the findings of the current study indicated that *P. pelagicus* and *P.sanguinolentus* crabs are extremely nutritious and protein-rich food items that are safe for eating by humans.

CONCLUSION:

According to the results of the current study, the marine crabs Portunus pelagicus and Portunus sanguinolentus can be recommended as ideal food items and can also be used as a supplement of protein and other nutritive matter to balance human nutrition and prevent nutritional deficiencies in the future.

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Table 1. Isolates with closest relative as identified by the 16S rRNA database from NCBI BLAST, query cover and per cent similarity, gram stain, motility test and colony morphology

Sequence	Closest relative	GenBank accession number	Cell morphology	Gram stain	Motility	similarity	Query cover (%)
MB1	*Enterobacter hormaechei*	ON248499	Straight rods	-	+	99.81%	100%
MB2	*Micrococcus caseolyticus*	ON248500	Cocci	+	+	99.35%	100%

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TABLE 2: Proximate composition of biomolecules

Sample	Protein (%)	Carbohydrate (%)	Lipid (%)	Moisture (%)	Ash (%)
Portunus pelagicus	23.6	3.6	0.5	60.31	11.40
Portunus sanguinolentus	25.3	1.3	2.3	55.06	10.52

Figure: 1; Graphical distribution of top 100 BLAST hits of *Enterobacter hormaechei*

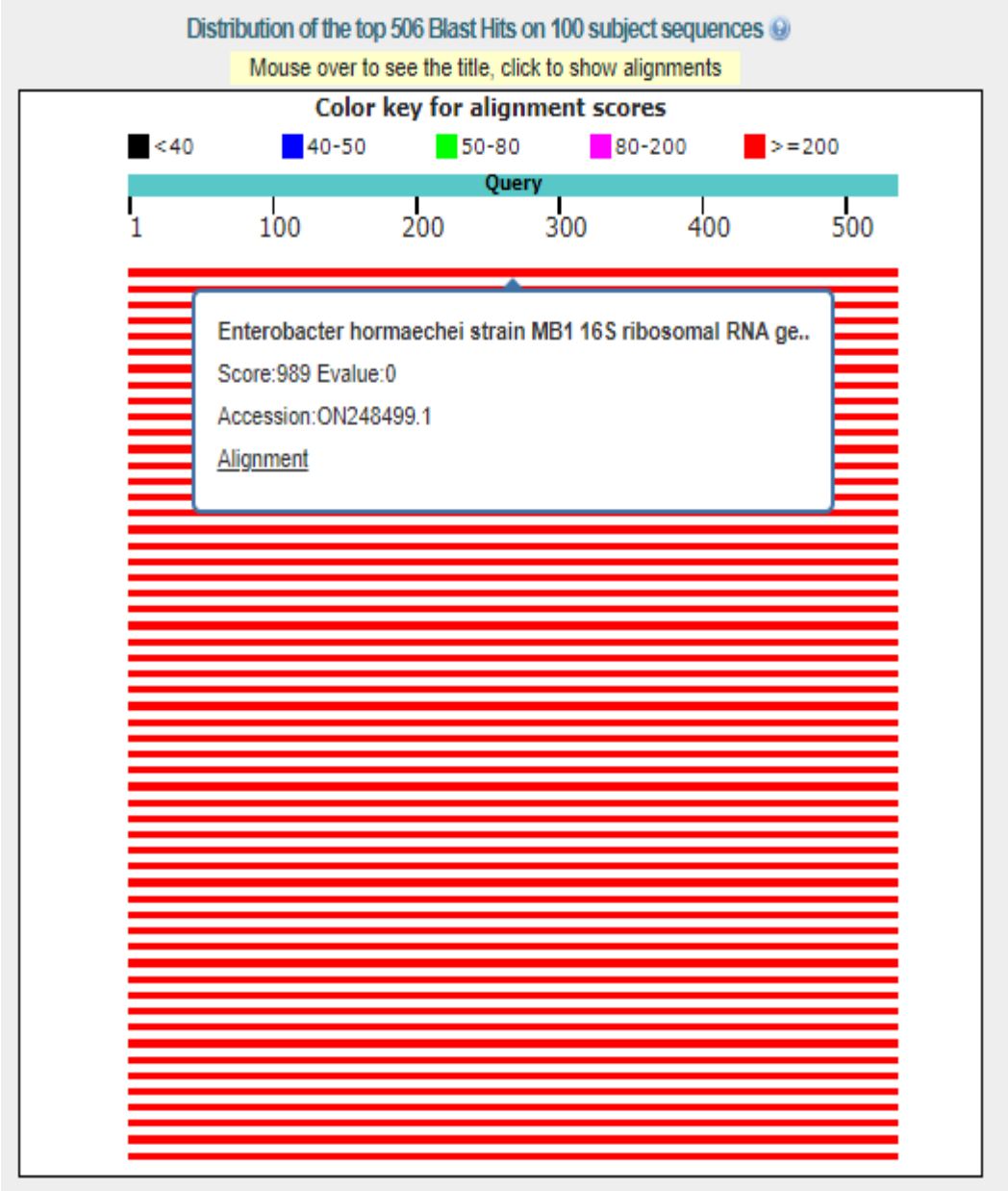

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Figure 2: Phylogenetic affiliations of the bacterial (*Enterobacter hormaechei*) 16S rRNA genes cloned from crab intestines

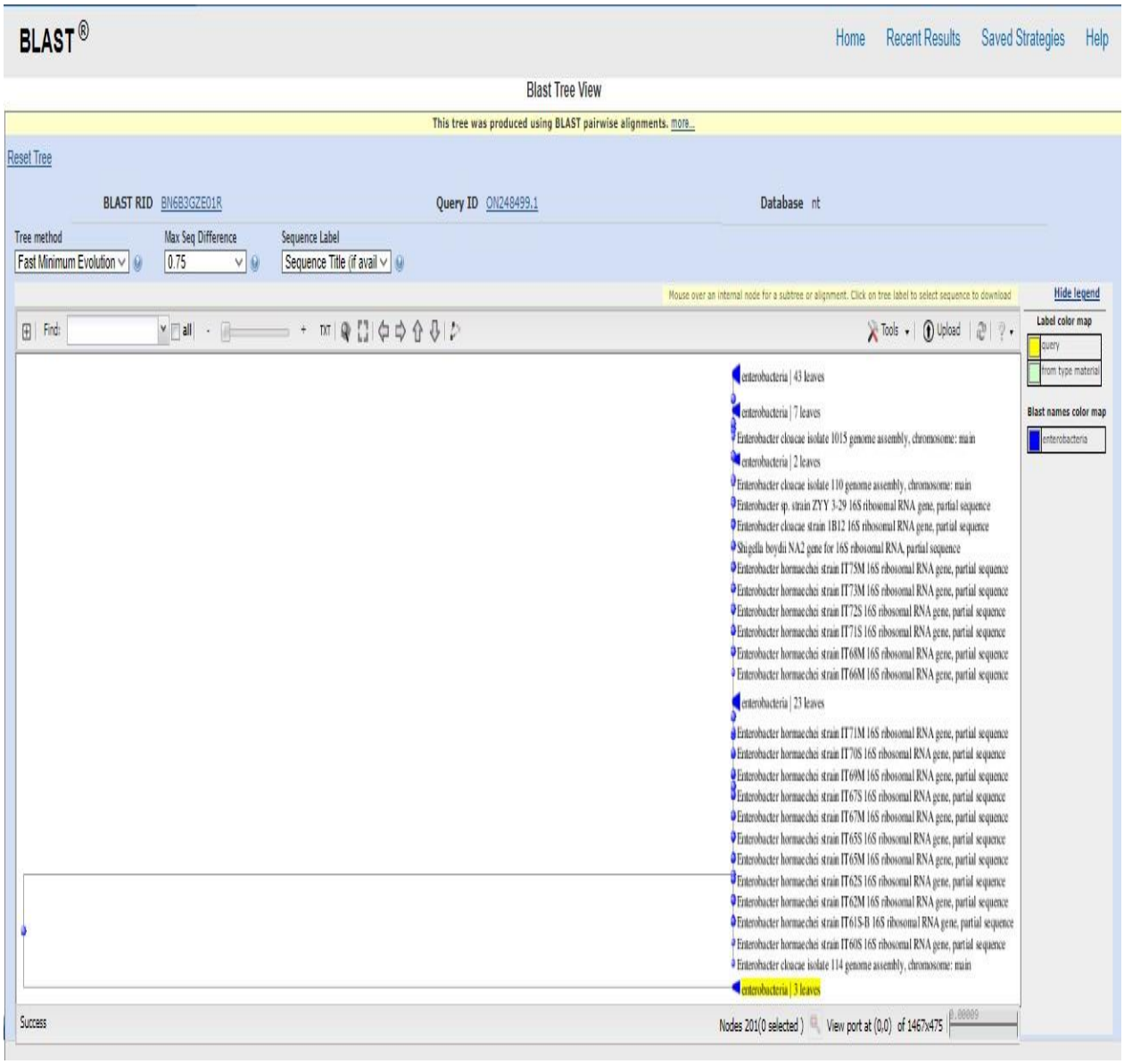

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Figure 3: Graphical distribution of top 100 BLAST hits of *Macrococcus caseolyticus*

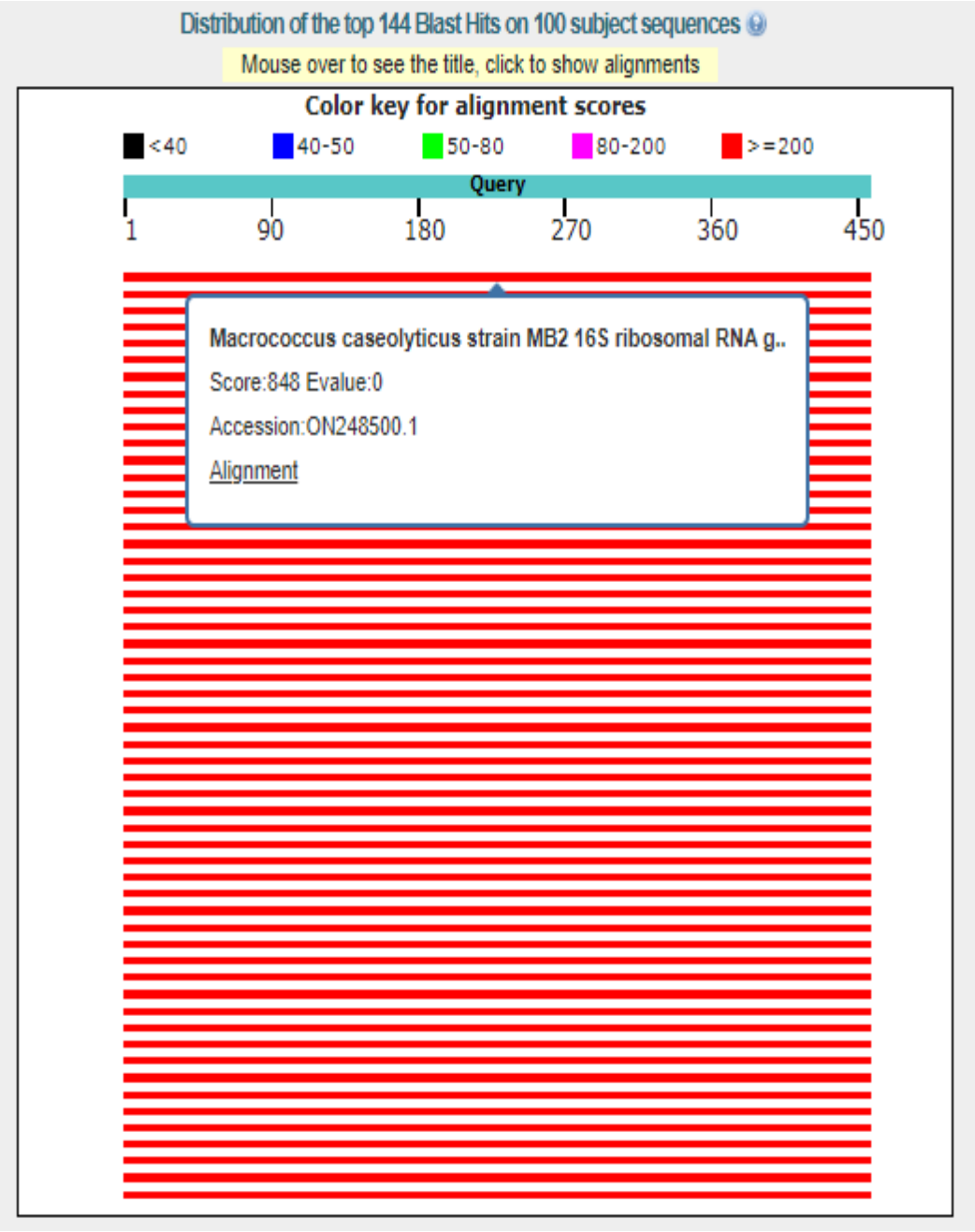

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Figure 4: Phylogenetic affiliations of the bacterial (*Micrococcus caseolyticus*) 16S rRNA genes cloned from crab intestines.

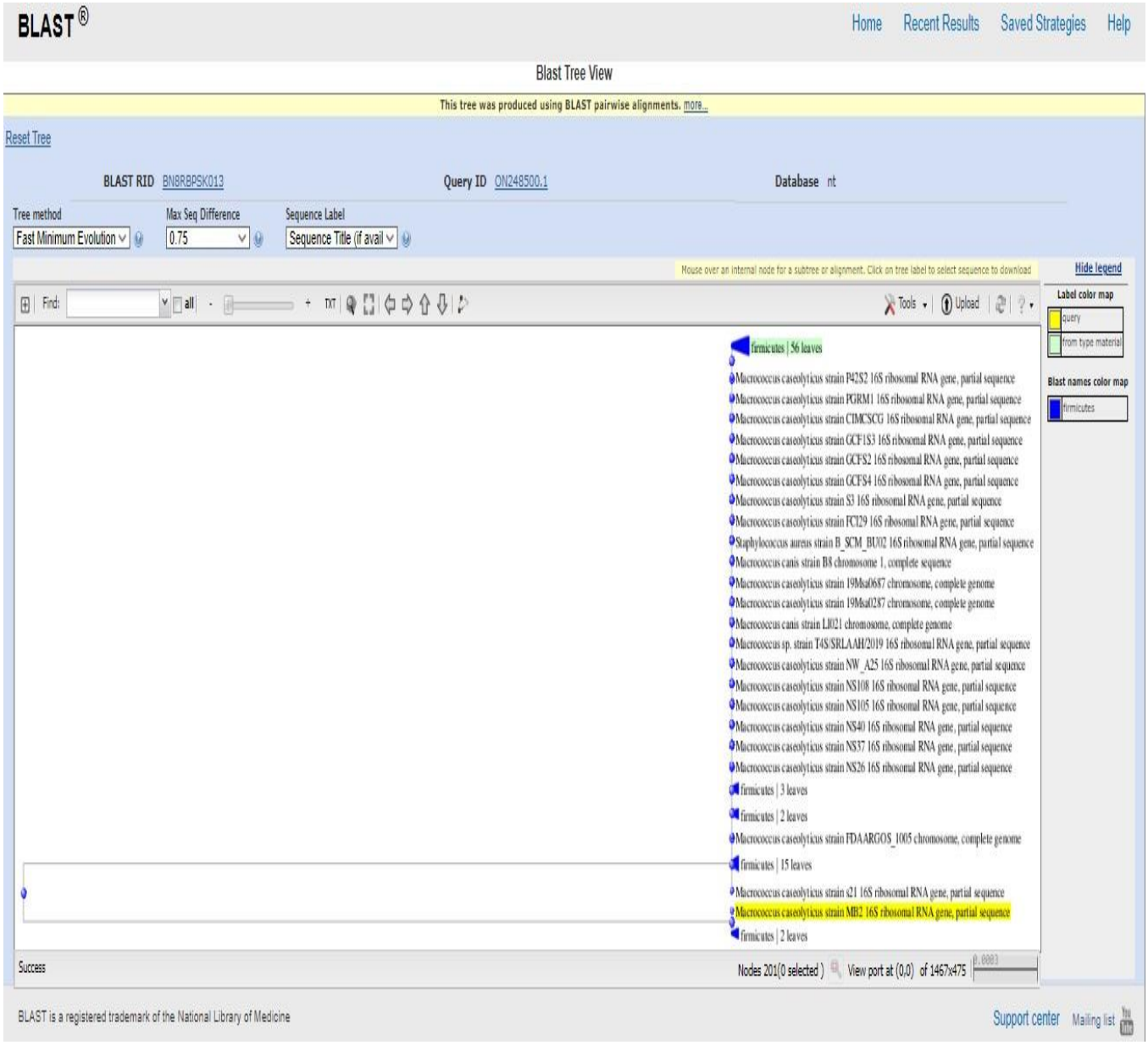

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FIGURE: 5 Chart shows the proximate composition of biochemical analysis