# CHAPTER-5

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## 5.1. Developmental parameters of *S. ricini* on Different food plants

The careful selection and effective utilization of food plants are pivotal in the sericigenous insect's physiological and economic well-being, given that their growth and development are predominantly reliant on the nutritional composition of their host plants. Three different categories of food plants for the study had been utilized and the data of the larval growth parameters assessed in the experiment showed significant differences among all three groups ($P<0.05$). The economic parameters of silkworm such as cocoon yield, shell weight and quality of silk are influenced by the type of food plant used for rearing silkworm larvae (Gangwar, 2010).

In present study, significant variations in the result have been observed in three larval groups each fed on different food plants. The *R. communis* fed larvae showed better larval parameters such as shortest larval duration, better larval length, larval weight and silk gland weight as compared to larvae reared on *M. esculenta* and *C. papaya* food plants. The better larval parameters on castor fed larvae had also been reported by earlier workers (Kumar and Elangovan, 2010; Kumar and Gangwar, 2010; Rajadurai *et al.,* 2010; Borah *et al.,* 2020). This indicated that castor served as the primary food for the proper growth and development of silkworm larvae. Kumar and Elangovan (2010) reported that the larval duration of castor leaves fed larvae was shortest (19.25 days) compared to larvae fed with the leaves of tapioca (20.50 days) and papaya (22.00 days). They also observed maximum larval weight in castor fed larvae (7.38 g) compared to tapioca (6.45 g) and papaya (5.55 g) food plants.

A similar trend of observations in larval weight and duration were also reported by Kumar and Gangwar (2010). The type of food plants and their nutritional quality also affect the survivability of insects feeding on them. Sarate *et al.* (2012) observed that larval growths are affected significantly by the quality of food plants.

Papaya plant leaves being tertiary food plant showed poorest growth parameters and lowest survivability rate in present investigation, suggesting that the papaya plant was not suitable for larvae in terms of growth and development. Similarly, the result of Konno *et al.* (2004) also reported high mortality of *S. ricini* larvae reared on papaya leaves. Their result clearly indicated that *C. papaya* leaves contain papain in latex which is responsible for strong toxicity against lepidopteran insects.

In the study, significant variations were observed in developmental parameters among larvae that were provided distinct diets consisting of castor, tapioca, and papaya may be attributed to the distinct diets provided. This aligned with the findings of He *et al.* (2021), they noted that host plants exhibit diverse nutritional compositions, palatability levels, and secondary metabolites, all of which possess the potential to influence the growth and developmental processes of *S. frugiperda* insects. The finding, underscored the importance of considering the impact of different host plants on insect development, emphasizing the multifaceted nature of the relationship between diet and developmental outcomes.

### 5.2. Proximate analysis of Food plants used for rearing of *S. ricini*

In subsequent investigation of the proximate composition of the food plants used for rearing of *S. ricini* silkworms *i.e.*, castor, tapioca, and papaya—the key parameters, including moisture, crude fibre, fat, carbohydrate, crude protein and ash content were systematically examined for significant variations ($P<0.05$). This analysis was conducted to elucidate the potential influences of host plant nutrient composition on the developmental parameters of the *S. ricini*.

The findings revealed substantial differences in the compositions of these plants, underscoring the diverse nutritional components inherent in each food source, which subsequently contributed to discernible variations in recorded larval growth parameters. This report was in agreement with the statement of Ravikumar (1988) who reported that the quality of leaves provided to silkworms for feeding is considered a primary factor affecting the production of high-quality cocoons. Variation in the proximate analysis of Eri silkworm's food plants was also reported by Deuri *et al.* (2017). Leaves with superior quality enhance the likelihood of producing good quality cocoons. In the present investigation, the crude protein of the larvae had been recorded at highest percentage among all other nutrients. Similar trend of result was reported by Longvah *et al.* (2011) and Hirunyophat *et al.* (2021) in which percentage of protein and fat content in dry weight of pre-pupae and pupae stage was comparatively higher than other nutrient components in Eri silkworm. This result may stem from the Eri silkworm larvae undergoing multiple transitional stages during their development, necessitating substantial energy and nutrient reserves. The high concentration of lipids and proteins in their bodies may allow them to sustain these growth phases and complete their life cycle successfully. The intricate nature of these outcomes highlights the nuanced and multifaceted relationship between the nutritional characteristics of host plants and their consequential impacts on the developmental aspects of the larvae.

### 5.3. Gut Digestive enzyme assay of *S. ricini* reared of three different food plants

Enzymes play a critical role in biological processes, and α-amylase, cellulase, proteinase, and lipase are essential enzymes with distinct functions. Amylases catalyses the breakdown of starch into simpler sugars like glucose, facilitating energy release during digestion. Cellulase, on the other hand, is crucial in breaking down cellulose, a complex carbohydrate found in plant cell walls, enabling the extraction of nutrients from plant-based foods. Proteinase is involved in the digestion of proteins, breaking them down into amino acids for absorption and utilization in various bodily functions. Lastly, lipase acts on fats, breaking them into fatty acids and glycerol, promoting fat absorption and energy release. The importance of these enzymes lies in their ability to enhance nutrient absorption, support energy metabolism, and contribute to overall digestive and physiological well-being. Without their activity, efficient nutrient utilization and the maintenance of essential bodily functions would be compromised.

In the comparative study of larval gut digestive enzymes activities, significant differences were observed in all the three sampled groups i.e. larvae reared on *R. communis* (Sample C), *M. esculenta* (Sample T) and *C. papaya* (Sample P) food plant leaves ($P<0.05$). Sample C showed highest α-amylase, cellulase and lipase activities than other two samples whereas lowest activities of α-amylase, cellulase and lipase were observed in Sample P. However, the Sample P also showed highest protease activity than the other two samples.

In present study, the higher activities of α-amylase, cellulase and lipase in Sample C was positively correlated with the better larval survivability whereas lowest activities of both the enzymes in papaya fed larvae was associated with poor larval growth parameters. Sample T showed moderate enzyme activities which was associated with moderate larval parameters. The higher activity of α-amylase may be associated with better digestion of carbohydrate rich leaves and better nutrient availability for the larval growth. This could have resulted in the better compatibility of Eri silkworm with castor leave feeding habit which directly related to better growth and development of silkworm larvae fed on castor leaves followed by tapioca and papaya. This result may be supported by the findings of (Mendiola-Olaya *et al*., 2000; Ahmadi *et al.,* 2012; Namin *et al.,* 2014). They found that the effectiveness of amylases are important for the survival of the insect and thus influences establishment of compatible insect-host relationship. Proteinase enzymes degrade the protein component of ingested diet and their activity depends on the protein content present in the leaves of host plants.

In the present investigation, gut proteinase activity was notably greater in Sample P in comparison to Samples T and C. Papaya leaf latex had been found to contain a significant amount of cysteine protease, called papain (Konno *et al*., 2004). The higher proteinase activity in the gut tissue of the larvae fed with papaya leaves might result from the plant's cysteine proteinase activity, particularly the papain enzyme found in papaya plant leaf latex. The response of the insect to the ingested proteinase inhibitors (PIs), a defence strategy of plants may result in hyperproduction of proteinase by the midgut cells (Namin *et al*., 2014; Holtof *et al*., 2019). Higher proteinase activity resulted in the higher larval mortality and the lowest values of larval growth index in papaya fed larvae. This finding may be supported by the work of Pechan *et al.* (2002) and Namin *et al.* (2014). The lipases play a crucial role in digestion of lipid components of ingested food materials into simpler absorbable form.

In the present investigation, higher lipase activity was recorded in Sample C. This result might be due to the variation in the level of lipid content present in the food plants. Deuri *et al*. (2017) reported significantly higher level of lipid content in the leaves of castor leaves (10.95%) than the tapioca leaves (0.22%). Lower lipid concentration in the ingested food might have resulted in lower digestive lipase activity in Samples T and P. Thus, it was evident that dietary contents exert crucial role in activity levels of digestive enzymes in the insect host. This could be supported by the work of Namin *et al*. (2014) who reported that the digestive enzymes activities are correlated with presence of different concentration of nutritional components and plant metabolites which in turn correlated with developmental parameters of the insects. Therefore, variation in the host plants can induce variations in the gut micro-environment subsequently digestive function of insects (Lee *et al.,* 2017).

### 5.4. Study of the diversity of bacterial communities in the larval gut of silkworm *Samia ricini* feeding on different food plants using a culture independent method

Insects gut bacterial study has gaining lot of attention with their increasing important role in various fields. Insects form mutually beneficial relationship with microorganisms either through inheritance or dietary acquisitions; these symbiotic partners may play a role in breaking down food at the cellular level and providing nutritional support to the host organism (Paniagua *et al*., 2018). Several researchers, especially those studying polyphagous insects, have documented the impact of diet on both the composition and functions of gut bacteria (Jones *et al.,* 2019; Majumder *et al.,* 2019; Leite-Mondin *et al.,* 2021; Mason *et al.,* 2021; Wang *et al.,* 2023).

Variation in the gut bacterial composition of Eri silkworm reared on three different food plants i.e., *R. communis, M. esculenta*, and *C. papaya* were observed. The findings of variation in the *S. ricini* gut bacterial community at every taxa level *i.e.* Phylum, class, order family, genus species, might be associated with their different food feeding and the diet change in host organism is the potent factors that largely influence the gut bacterial composition (Dong *et al.,* 2018) as individual food plants contribute differently within the gut microenvironments, subsequently impacting the diversity of its inhabitants gut microorganisms (Lee *et al*., 2017). Similar finding was reported by Han *et al.* (2023) who recorded variation in the diversity of gut bacteria in *S. frugiperda* larvae reared on different host plants. Chen *et al.* (2018) described the relative association of diversity and composition of gut flora with host plants, developmental stage and physiological conditions of the host silkworm. Colman *et al*. (2012) also confirmed the influences of diet and host's taxonomy on gut microbial composition.

Gut bacterial population are believed to complement the host by performing essential roles directly or indirectly benefiting its host. In this study, gut bacteria belonging to the phyla *Proteobacteria* and *Firmicutes* exhibited dominance consistently across all taxonomic levels, including Phylum, Class, Order, Family, Genus, and Species. Also the relative abundance found varied among the all the three groups *i.e.* Sample C, T and P, with the highest percentage recorded in the larvae reared on the primary castor food plant compared to the secondary food plant, tapioca, and the tertiary food plant, papaya.

Similar observations were reported by MsangoSoko *et al.* (2020b), they reported *Proteobacteria* and *Firmicutes* as dominant phyla in Eri silkworm represented with 60% and 20% of total sequences respectively. In a related study in lepidopteran pest, *Spodoptera exigua*, the gut microbiota of larvae was recorded to consist of 97.9% *Proteobacteria* and 2.1% *Firmicutes* (Presa-Parra *et al.,* 2020), indicating the prominence of these two phyla in lepidopteran insect species. However, the variation in the percentage of gut bacterial phylum that had been recorded in this study and certain alteration might be due to different food plants that been used for feeding the silkworm. As, dietary patterns in insects play a crucial role in shaping the structure and composition, as well as the diversity and function, of bacterial communities within the insect gut (Semova *et al.,* 2012), which may be essential in long-term adaptation to host plants and individual host (Wu *et al.,* 2019; Yuan *et al.,* 2021).

Numerous investigations have recorded that *Proteobacteria* and *Firmicutes* prevail as the primary bacterial phyla in the bacterial communities within the gut of insects, particularly in Lepidopterans (Sharon *et al*., 2010; Chen *et al*., 2018; Chen *et al*., 2020; González-Serrano *et al*., 2020; Zhang *et al.,* 2020). In an investigation conducted by Tinker and Ottesen (2016), it was revealed that the composition of gut bacteria in cockroaches is influenced by the dietary substrate which in turn alterations in the food source, resulted in modifications to the nutritional matrix accessible for gut bacteria, ultimately causing shifts in the gut flora. Similarly, as observed by Wang *et al.* (2023), the significant microbial differences were recorded in *S. frugiperda* larvae fed on various hosts which could be attributed to the differing nutritional content and the presence of inhibitory secondary metabolites in the four hosts used for feeding.

The crucial roles played by the members of phylum *Proteobacteria* and *Firmicutes* have been demonstrated by different researchers in terms of nutrients digestion and absorption, metabolism and protection of the host against diseases in insects (Anand *et al.,* 2010; Feng *et al.,* 2011; Bhuyan *et al.,* 2018; Unban *et al.,* 2022). In Sample C, the finding of a higher relative abundance of both dominant phylum (*Proteobacteria* and *Firmicutes*), may have contributed to enhanced nutrient acquisition and provided increased protection to the larvae, resulting in overall healthier larval development compared to Samples T and P. This finding could be well supported by the report of Douglas (2015) that gut microbes establish symbiotic relationships with hosts, residing in various parts such as exoskeletons, guts, and even within cells, often proving beneficial or vital for the insects' survival. The roles of *Proteobacteria* and *Firmicutes* symbionts extend beyond all these they also have been documented to actively participate in the digestion and utilization of a range of polysaccharides, including cellulose and hemicellulose (Anand *et al.,* 2010; Engel *et al.*, 2012). In the lower taxa, at the genera level most abundant genera identified in all the three groups with varying percentage and relative abundance were *Enterobacter, Enterococcus, Klebsiella* and *Lactobacillus*.

Among larvae fed on castor (Sample C), the most dominant genus was *Enterococcus*, followed by *Klebsiella*. In contrast, Samples T and P showed dominance of *Enterobacter* and *Enterococcus*, with *Enterobacter* exhibiting the highest relative abundance in Sample T. The observed variations in bacterial community composition may be attributed to the distinct nutritional profiles and microbial environments associated with different host plants, influencing the prevalence of specific bacterial genera in the larvae. In essence, the gut microbiota can be affected by the diet, both through direct and indirect process (Douglas, 2015; Liu *et al.,* 2020). About 40% of the OTUs were unknown at the genus level. The presence of *Enterobacter* and

*Enterococcus* as dominant genera had been previously reported in silkworm gut (Liang *et al.,* 2014; Chen *et al,* 2018; MsangoSoko *et al*., 2021). Broderick *et al.* (2004) reported that *Enterobacter* and *Enterococcus* occurs most prominently in the larval gut of gypsy moth reared on varying diets and both are recognized as the predominant intestinal bacterium within the order Lepidoptera (Wu *et al*., 2019; Presa-Parra *et al.,* 2020; Yuan *et al.,* 2021). The crucial role of *Enterobacter* in glucose metabolism and cellulose degradation has also been reported by Rezaei *et al.* (2009) and Yuan *et al.* (2021).

Previous studies have found that gut bacteria like *Enterobacter* and *Enterococcus* isolated from a lepidopteran insect complement the host by regulating the functions such as host defence against pathogens, nutrition, metabolism (Producing amino acids, lactic acid, metabolites), production of digestive enzymes, regulation of gut pH, tannin tolerance (Anand *et al.,* 2010; Liang *et al.,* 2014; Sun *et al.,* 2016; Hou *et al.*, 2018; Unban *et al.,* 2022;).

Furthermore, the increased prevalence of *Enterococcus* is believed to contribute in enhanced digestion and the expedited development of resistance to insecticides (Lu *et al*., 2023). Additionally, role of *Enterococcus* in stabilizing the host insect's tolerance efficiency to toxic diets by degrading alkaloids and latex were also reported by Liu *et al*., (2020). The genus *Klebsiella*, within family *Enterobacteriaceae* has also been widely documented in the digestive systems of Lepidoptera and other herbivores, indicating its potential as a beneficial and non-pathogenic microorganism (Chen *et al.,* 2016; Chu *et al*., 2013; Suen *et al*., 2010). The role of genus *klebsiella* in cellulose degradation, immune system enhancement, and support for the growth and development of insects was again robustly reported by Ami *et al.* (2010); Hamden *et al*. (2013); Dantur *et al.* (2015); Muhammad *et al.* (2017).

Therefore, the presence of these dominant genera in the gut of Eri silkworm reared on three different food plants might have exerted their beneficial impact on host differently. This implied a mutually advantageous association between the host and these reliable microbial partners, furnishing a steady and consistent source for the metabolic processes of the host insect. This observation was corroborated by the reports of Shin *et al.* (2011) and Douglas (2015). The digestive systems of the majority of insects accommodate a varied community of commensal microorganisms, participating in a mutually beneficial symbiotic relationship with the insects through extensive co-evolution, as emphasized in these studies.

At the lower taxa level some unknown bacterial were recorded. At the species level a huge fraction of the total OTUs (89.18%) were unknown and only 10.82% OTUs had been identified including uncultured and some other known bacterial species (Figure 4.7.6). The presence of these unclassified taxa in the gut sample could be possible due to the limitations in the reference databases, as diversity studies rely on the existing databases to classify the bacteria. Limitations in methodology for accurate identification, evolutionary novelty or some of the bacteria found in insect gut may be new that are not closely related to known bacterial taxa (Yun *et al*., 2014). This was in agreement with the findings of earlier researchers (Jovel *et al*., 2016; MsangoSoko *et al*., 2020b), who reported the occurrence of unassigned taxa and concluded that such observations could be due to appearance of novel microbes or failure of resolution as targeting only V3-V4 region of 16S rRNA would not be able to resolve the proper taxonomic annotation.

Furthermore, co-occurrence analysis of the gut sample revealed that several bacterial genera, were found to co-occur together in the larval gut of *S. ricini*. In all the three groups it was seen that the genus *Klebsiella*, *Enterococcus*, *Serratia*, *Bacillus* and *Staphylococcus* were present as core bacterial genus in the gut of Eri silkworm larvae. This indicated that these gut bacterial genera might have co-existed in the gut tissue either due to common habitat preference or shared resources or mutual interactions within the gut of the eri silkworm reared on tapioca food plants.

The co-occurrence network analysis of gut bacterial genera in the three samples (C, T, and P) showed variation in the intricate microbial composition within the gut of Eri silkworm larvae. In Sample C, where larvae were fed with Castor leaves, a diverse set of highly connected bacterial genera was identified, including *Klebsiella,* *Enterococcus, Enterobacter, Delftia, Serratia, Burkholderia-Caballeronia-Paraburkholderia, Pseudomonas, Bacillus,* and *Staphylococcus*. This suggested a complex interconnection among these genera in response to the specific diet. In Sample T, notable bacterial genera such as *Lactobacillus*, *Enterococcus, Lactococcus*, and others dominated the connectivity network. Interestingly, *Bacillus, Stenotrophomonas, Staphylococcus, Serratia*, and *Klebsiella* were also prevalent, hinting at potential functional roles and interactions within the larval gut environment. In contrast, Sample P displayed a distinctive set of core bacterial genera, including *Staphylococcus, Enterococcus, Enterobacter,* and others. The presence of *Brevundimonas, Acinetobacter*, and *Klebsiella* further added to the complexity of the gut microbiota. Across all three samples, the consistent presence of dominant genera *Klebsiella, Enterococcus, Bacillus*, *Serratia,* and *Staphylococcus* as core bacterial genera suggests their fundamental roles in the gut ecology of Eri silkworm larvae.

The observed variations in bacterial composition among samples may be attributed to dietary differences, indicating the influence of diet on shaping the microbiome and highlighting the functional roles of these microbial dynamics in the context of Eri silkworm development and health. This finding aligned with existing research emphasizing the significance of *Klebsiella*, *Enterobacter*, and *Bacillus* as symbiotic bacteria in various vital functions. Previous studies have reported their roles in host adaptability, sexual performance, tolerance, and degradation of toxic plant materials, enhancement of immunity, acceleration of growth, carbohydrate degradation and metabolism, host metabolism, production of a wide range of digestive enzymes, and overall promotion of the host's growth and development (Ami *et al*., 2010; Hamden *et al*., 2013; Dantur *et al*., 2015; Muhammad *et al*., 2017; Paniagua *et al*., 2018; Zhao *et al*., 2018; Liu *et al*., 2020; Fatani *et al*., 2021; Yuan *et al*., 2021; Lu *et al*., 2023). This extensive information provides strong support for the functional roles of these key microbial dynamics in the context of Eri silkworm development and health.

The chao1 metric estimates the species richness while Shannon metric is the measure to estimate observed OTU abundances, and accounts for both richness and evenness. The observed species metric was the count of unique OTUs identified in the sample. All the three curves showed that the evenness of diversity was achieved in almost all samples. The microbial diversity analysis employing Shannon, Chao1, and observed species metrics had provided valuable insights into the composition and distribution of species within the samples. Notably, the Shannon metrics unveiled that Sample-T exhibited superior species richness and evenness compared to both sample-P and sample-C. This suggested a more balanced microbial community in the tapioca sample. Conversely, the Chao1 metric results highlighted a distinctive pattern, indicating that Sample-P harbours a significantly higher number of unique Operational Taxonomic Units (OTUs) compared to both Sample C and Sample T.

This observation highlighted the presence of distinct microbial taxa in papaya, contributing to its unique microbial profile. Moreover, the rarefaction curve analysis of observed species demonstrated a compelling trend. Sample P exhibited the highest number of observed species, with a continual increase in species count with each advancing x value. In contrast, Sample T and Sample C followed with decreasing levels of observed species. This implied a diverse and expanding microbial community in papaya, while tapioca and castor show comparatively less dynamic microbial populations. These discoveries collectively emphasized the intricate microbial dynamics observed within the samples, providing valuable insights into the diverse and unique characteristics inherent in each. This diversity in microbial composition may be intricately linked to the significance of food plants, suggesting their influence on the gut microenvironment and subsequent variations in gut bacterial populations across the three samples. Notably, Sample P exhibited a more diverse microbial population compared to Sample C and Sample T, both of which demonstrated relatively similar levels of gut bacterial diversity. This result aligned with their roles as primary and secondary food plants, respectively, while papaya, being a tertiary food plant, showcased a heightened microbial diversity. This observation could be supported by the phylogenetic tree of gut bacterial populations among samples, demonstrating variations (Figure 5.1). Notably, Samples C and T exhibited a degree of similarity in bacterial composition, with a 63% resemblance. However, Sample P displayed distinct bacterial populations compared to both Samples C and T, being grouped into a different clade.

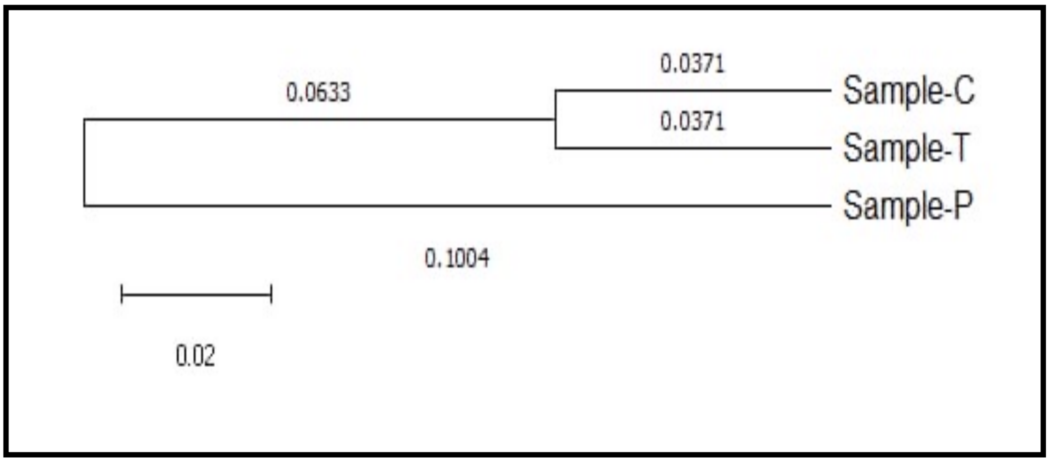

Figure 5.1. Phylogenetic relationships of gut bacterial population of three samples

In the Principal Coordinate Analysis (PCoA) of the samples (Sample C, Sample T, and Sample P), variations was depicted in the PCoA plot. This finding was consistent with the diversity analysis results, indicating that the observed variations may imply a dynamic shift in both the structure and diversity of gut microbial communities within the *S. ricini* larvae feeding on different food plants. These findings may be supported by the work of Lee *et al.* (2017), who noted that different host plants can induce variations in insect gut microenvironments, ultimately leading to differences in gut microbial diversity. Similar observation was reported by Jones *et al.* (2019) who demonstrated that host plants are significant drivers influencing the gut microbiota of polyphagous insects. Leite-Mondin *et al.* (2021) study also supports the present findings by noting alterations in gut bacterial species diversity and richness in polyphagous *Trichoplusia ni* insects in response to dietary changes.

Similarly, recent studies by Mason *et al.* (2021) and Wang *et al.* (2023) provide additional corroboration, emphasizing the impact of diet on gut microbial communities. Mason *et al.* (2021) highlighted variations in the gut microbiome of *Spodoptera frugiperda* feeding on different plant species, affirming the influence of dietary factors on microbial composition and diversity. Wang *et al.* (2023) further confirmed the importance of host plants by demonstrating their significant effects on the structure and diversity of gut bacterial communities in Lepidopteran insect *S. frugiperda*, aligning with the findings that different food plants contribute to the observed variations in microbial richness and uniqueness across the samples. The alignment between the results and recent studies emphasizes the crucial influence of host plants in shaping gut microbial communities.

In further exploration to identify the shared and unshared bacterial Operational Taxonomic Units (OTUs) through a Venn diagram and Sorenson's similarity index calculation between samples, a higher prevalence of shared OTUs was observed between Sample C and Sample T in comparison to Sample C and Sample P, as well as Sample T and Sample P. This divergence could be attributed to the distinct feeding habits of the larvae associated with each sample, suggesting an influence of the nature and category of the respective food plants. These findings aligned with earlier analyses in this study, specifically the assessments of gut bacterial community structure and diversity. Significantly, analysis of gut bacterial community structure revealed a heightened percentage of unknown or unclassified bacterial OTUs in Sample P in contrast to Samples T and C. This disparity may be linked to the unique impact of papaya leaves on the gut of *S. ricini* silkworm larvae, serving as a tertiary food source that contributes differently compared to the commonly used secondary foods, castor and tapioca leaves. This observation underscores the importance of considering the nature of food plants, the secondary metabolites they contain, and the nutritional needs of the host organism in understanding the composition of the gut microbiota, as highlighted by Yang *et al.* (2020).

The work of Zarraonaindia *et al.* (2015) provided evidence that the environmental microbiome, influenced by factors such as rain splash or wind, can enrich the leaf microbiome of different host plants, which in turns plays a role in shaping the gut bacterial community structure as they are consumed by the host insect species. The present findings resonated with prior research. Furthermore, researchers had validated that significant changes in diet can deeply impact the composition of the gut microbiome (Perez-Cobas *et al*., 2015; Erkosar *et al*., 2018). Similarly, Mason *et al.* (2020) documented that diverse diets exert an influence on the multiplication of gut microbes in *S. frugiperda* insects.

Therefore, the observed differences in gut bacterial community structure, richness and uniqueness among the samples can be attributed to the hierarchical roles of these plants in the food chain, affirming the intricate interplay between plant types and microbial communities. This comprehensive analysis provided valuable insights into the nuanced dynamics governing the gut microbiota of *S. ricini* larvae in response to different food sources.

To unveil the functional capabilities of gut bacteria communities in Eri silkworm reared on different food plants we further analysed comparative KEGG pathway analysis using software Picruist2 using 16 S rRNA sequencing data in 3 levels to understand their possible biological roles. The variation in the percentage of gut bacterial genome involved in various physiological roles was recorded in all three groups. This result might indicate that roles of bacteria also get influenced by the diet of host organism which might help the host organism to adapt on the experimental diet. Gut bacteria are known to be highly adaptable to change in their diet as well as environmental conditions (Yun *et al.,* 2014; Beam *et al.*, 2021). In the first level of analysis cluster of Orthologous groups (COG) was compared and identified.

The analysis of *S. ricini* gut bacteria using KEGG pathway annotations at Level 1 revealed six major functional pathways: metabolism, environmental information processing, genetic information processing, cellular processes, organismal systems, and human diseases. The role in metabolism was predominant across all samples of Eri silkworm larvae. Specifically, in the Metabolism pathway, Sample T exhibited the highest percentage, followed closely by Sample C and then Sample P. Environmental Information Processing and Genetic Information Processing pathways also showed variations among the samples, while Human Diseases and Organismal Systems pathways exhibited minimal variations, indicating a relatively consistent distribution across all three samples. This finding indicated that gut bacterial communities in the gut not just inhibit inside the gut of Eri silkworm but also inhibit as a gut symbionts of host insect that complements its host and may help in host physiological roles particularly involved in host metabolism. This result indicated that these conservative bacterial communities could help herbivorous insects adapt to the host and play an important role in physiological metabolism (Li *et al.,* 2022c). However, no previous reports existed regarding the functional annotation of gut bacterial communities and the impact of food plants in gut community composition in Eri silkworms, making it challenging to draw comparisons with the present investigation.

Further exploration at Level 2 delved into the distribution of functional gene categories within the gut bacteria of samples T, P, and C across various KEGG pathways. Remarkably, the Membrane Transport pathway displayed variations, with sample C leading, followed by P and then T. Carbohydrate metabolism, amino acid metabolism, and replication and repair showed trends across the samples, while Energy Metabolism, Translation, and Cellular Processes and Signalling pathways exhibited variations. Nucleotide Metabolism and Metabolism of Co-factors and Vitamins demonstrated subtle differences, emphasizing sample-specific functional characteristics. This result clearly indicated the importance of gut bacteria in essential metabolic roles of gut bacteria which might be related in contributing the healthy health of host silkworm supported by findings of Li *et al.* (2022a). As gut symbionts were involved in metabolism of silkworm which enhanced the health status of host silkworm. Similar observation was reported on the other Lepidopteran insect (*S. frugipenda*) in a recent work by Wang *et al.* (2023). They also reported six orthologous class of function with highest functional prediction on the role of metabolic pathways with highest percentage in membrane transport, carbohydrate metabolism and amino acid metabolism varying proportion of pathways on insect on different host plants.

In another study by Yuan *et al.* (2021) in *Grapholita molesta* (Lepidoptera) reared on different host plants. They also recorded dominant role of gut bacteria mainly in transport and metabolism of Amino acid, Carbohydrate, Translation, ribosomal structure and biogenesis and, Nucleotides, which was consistence with present findings. This findings suggest that in lepidopteran insects gut bacteria are may be dominantly associated with roles in transports and metabolism of particularly essential nutritional and essential molecules such as carbohydrate, amino acid, vitamins and cofactors, nucleotides and other essential metabolism. This may be supported by the finding of other researchers that Various microorganisms associated with insects contribute particular nutrients that insects are unable to produce independently, including crucial amino acids (Feldhaar *et al.,* 2007; Tokuda *et al.,* 2013) and B vitamins (Shigenobu *et al.,* 2000; McCutcheon *et al*., 2007). This discovery implied the potential complementary functions of gut bacteria in Eri silkworms raised on diverse food sources.

However differences in the percentage of predicted roles in the gut bacteria of Eri silkworm on different food plants might be contributed by the different food plants that have been used for feeding as similarly recorded by Wang *et al*. (2023) and Yuan *et al*., (2021). This could also be related to proximate Analysis. Present study had recorded differences in the proportion of essential nutrients in all the three plants. This finding may be justified by the finding of researchers that recorded that the host's carbohydrate metabolism, amino acid metabolism, and membrane transport pathways are significantly influenced by the pivotal involvement of intestinal bacteria (Wang *et al*., 2020; Genta *et al.,* 2006; Warnecke *et al*., 2007). Such functions are likely indispensable for insects to successfully adjust to distinct host plants (Yang *et al.,* 2020).

Furthermore at Level 3, the analysis revealed enrichment of different functional proteins in the guts of fifth instar Eri silkworm larvae reared on different host plants. The proteins included peptide/nickel transport system permease protein, ATP binding proteins, substrate binding proteins, iron complex outer membrane receptor protein, as well as enzymes like F420H(2)-dependent quinone reductase and transketolase. The statistical analysis identified significant variations in the proportion of functional proteins among groups. Peptide/Nickel Transport System Permease Protein exhibited distinct prevalence in Sample P, indicating unique protein expression profiles in each sample.

However, present study have also recorded role of gut bacteria in Xenobiotoxic degradation in all the three groups with high percentage in both Sample T and Sample P and slightly lower in Sample C. Role in immune responses also recorded higher in Sample C however very low percentage of role was observed in both Sample T and P.

This variation in the accumulation of genes associated with detoxification and immune responses in the *S. ricini* gut could be linked to the variety in food consumption or the fluctuating host environment (Wang *et al.,* 2023). These findings clearly indicated the influence of individual food plants on functional roles of gut bacteria as postulated that role of gut bacteria is also dependent on the quality and composition of host plants leaves.

Moreover, present investigation into *S. ricini* reared on different food plants revealed distinct enrichments of functional proteins associated with various metabolic pathways. Particularly noteworthy were the significant variations in the expression levels of key proteins observed across three samples. The Peptide/Nickel Transport System families, including peptide nickel transport system permease protein and substrate binding proteins, exhibited a higher proportion in Sample P compared to T and C. The prevalence of peptide/Nickel transport system protein families in Sample P, with a significantly higher proportion of sequences compared to other samples, suggested a distinct microbial response or adaptation in the gut of Sample P. This may be associated with the malnutrition in the larvae (Park *et al*., 2023), as nutritional content of food plants vary in different food plants. This could be demonstrated by the function of Nickel transport systems which are essential for the uptake of nickel, which can serve as a cofactor for various enzymes. Peptide transport is crucial for acquiring nitrogen-containing compounds, which are essential for the synthesis of nutrient compounds (Mulrooney and Hausinger, 2003).

Other functional protein F420H(2)-dependent quinone reductase and sucrose 6 phosphatase exhibited notable variations, with sample T demonstrating the highest expression levels. The differences in percentage proportions between samples indicated sample-specific variations, contributing to a dynamic and distinct functional protein expression pattern according to adaptation of insect on diet. F420H(2)-dependent Quinone Reductase enzyme is involved in the reduction of quinones, compounds often involved in electron transfer reactions. F420H(2)-dependent quinone reductase may plays a role in microbial metabolism, particularly in redox reactions (Bruggemann *et al*., 2000). The prominent variation in the expression levels of this enzyme across samples indicated differential redox activities in the gut microbiome. Higher expression in Sample T suggested a potential impact of the host plant Tapioca on the redox state of the microbial community. Sucrose 6-phosphatase is an enzyme involved in the metabolism of sucrose, a disaccharide sugar, which catalyses the hydrolysis of sucrose-6-phosphate to free phosphate and fructose (Hawker and Hatch, 1966). The differential expression of sucrose-6-phosphatase across samples reflects variations in sucrose metabolism within the gut microbiome. Higher expression in Sample T implied a potential influence of the host plant Tapioca on the utilization of sucrose.

The study also identified important proteins like heat shock protein, Lac I transcription regulatory protein, ATP binding protein, Iron complex transport protein, and polar amino acid transport proteins, with varying gene involvement across all three groups. Similar functional proteins were noted in a study by Wang *et al.* (2023) in another lepidopteran gut bacterial functional predictions analysis. These findings underscored the significance of predicted pathways in the gut, implying their crucial roles in the overall growth and development of the host insect.

In this study, the multi-level KEGG pathway analysis provided a comprehensive understanding of the functional potential of *S. ricini* gut bacteria. The differential expression of functional proteins across samples highlighted the impact of host plants on the gut microbiome, emphasizing the intricate interplay between microbial communities and their environment.

### 5.5. Study of nutritionally beneficial gut bacteria through culture dependent approach

Herbivorous insects possess various morphological, behavioural, and physiological attributes that help them to adapt and overcome dietary challenges (Hood *et al.,* 2015; Chaturvedi *et al.*, 2017; Chen *et al.*, 2021). Some insects can adapt to new host plants, and in that process, changes occur in the abundance and composition of gut enzymes. Many researchers (Anand *et al.,* 2010; Feng *et al.*, 2011, Bhuyan *et al.*, 2018; Liu *et al.*, 2018; Gandotra *et al.,* 2018b; MsangoSoko *et al.*, 2020 a and b; MsangoSoko *et al.*, 2021; MsangoSoko *et al.*, 2022) have already demonstrated the role of gut bacteria in digestive enzyme activity of insects including the silkworms. In this study, the isolated gut bacteria from *S. ricini* reared on three different food plants (castor, tapioca, and papaya) were analysed to comprehend and authenticate the role of gut bacteria in the production of some digestive enzymes. Additionally, this study aimed to investigate the impact of each individual plant on the levels of various enzyme activities.

In the qualitative screening of gut bacterial isolates from the *S. ricini* reared on castor, tapioca, and papaya, a total of 14 bacterial isolates exhibiting enzymatic activity were identified. In sample C among the digestive enzyme producing bacterial isolates *M. sciuri* (OR016518) and *Mammaliicoccus sp.* (OR828234) dominated the bacterial profile, suggesting *Mammaliicoccus sp.* prevalence. The *B. licheniformis* (OR739576), *Winslowiella sp.* (OR963258), *Brevundimonas diminuta.* (OR964999), and *Staphylococcus sp.* (OR211560) were also identified, indicating a diverse bacterial community. Sample T exhibited a different microbial landscape, with *Mammaliicoccus sp.* (OR921980) and *Bacillus sp.* (OR976063) as prominent members. The *B. subtilis* (OR923392) and *Mammaliicoccus sciuri* (OR924301) contributed to the diversity of bacterial species in this sample. The prevalence of *Bacillus* species sets sample T apart from Sample C. In contrast, Sample P displayed a unique bacterial composition. *W. iniecta* (OR945735), *K. oxytoca* (OR958639), *Citrobacter sp.* (OR976270), and *Mammaliicoccus sp.* (OR958729) were the identified species. Markedly, *Winslowiella* and *Klebsiella species* distinguished Sample P from the other samples, contributing to its distinct microbial profile. These findings underscored the microbial diversity among Samples C, T, and P. While sample C was characterized by a prevalence of *Mammaliicoccus species*, Sample T exhibited a significant presence of *Bacillus species*. Sample P, on the other hand, was distinguished by the presence of *Winslowiella* and *Klebsiella species*, emphasizing the unique bacterial composition in each sample. Further Physiological characterization of bacterial isolates highlighted the diverse traits among the bacterial isolates from samples C, T, and P, providing valuable insights into their functional capabilities and potential ecological roles.

These isolates were distributed among three distinct groups of *S. ricini* based on their respective diets: six isolates later were identified as (C1- *Mammaliicoccus sciuri*, C2- *Mammaliicoccus sp.*, C3- *Bacillus licheniformis,* C4- *Winslowiella sp.*, C5- *Brevundimonas diminuta*, C6- *Mammaliicoccus sp.*) from Sample C, four isolates (T1- *Mammaliicoccus sp.*, T2- *Bacillus sp.*, T3- *Bacillus subtilis,* T4- *Mammaliicoccus sciuri*) from Sample T, and four isolates (P1- *Winslowiella iniecta*, P2- *Klebsiella oxytoca*, P3- *Citrobacter sp*., P4- *Mammaliicoccus sp.*) from Sample P. The quantitative analysis of enzyme's assay of isolates has unveiled noteworthy variations in the digestive enzyme activities among gut bacterial isolates of the *S. ricini* reared on three distinct food plants *i.e.* castor, tapioca and papaya (Figure 5.2).

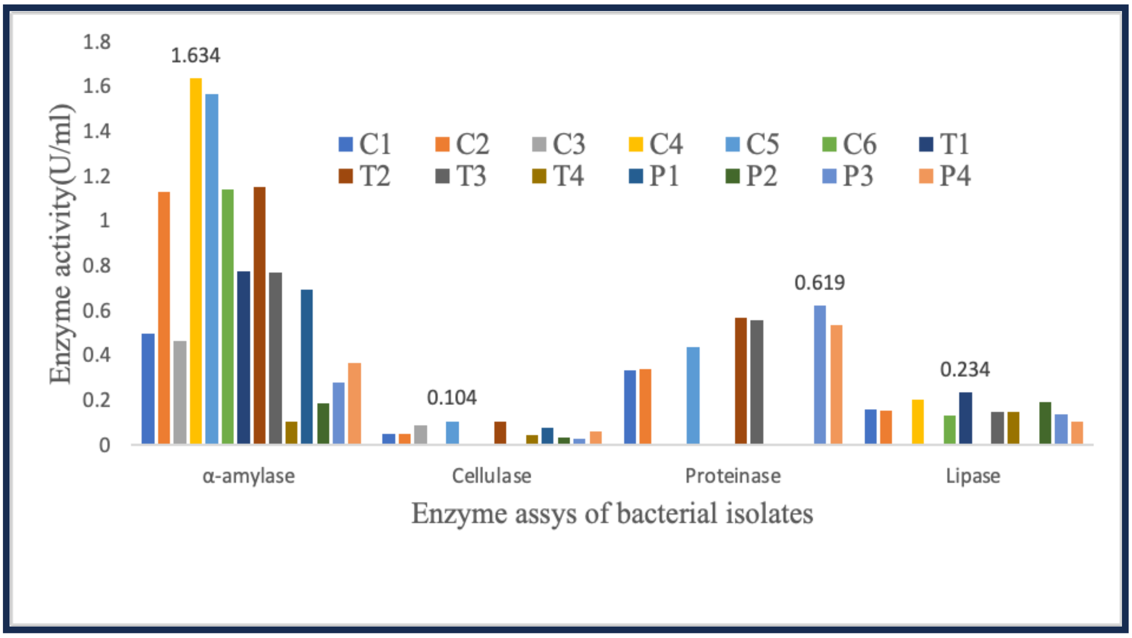

Figure 5.2. Digestive enzyme activities of gut bacterial isolates from three samples

The isolates were analysed for their amylase, cellulase, lipase, and proteinase activities, providing a comprehensive view of their digestive capabilities. α-amylase activity was recorded in all the 14 isolates, among the isolates, the maximum isolates exhibited substantial roles were observed in the α-amylase activity category with highest from Sample C, a range of 0.276-1.634 U/ml alpha amylase activity was recorded in enzyme assay with a subset exhibited remarkable amylase activity, primarily represented by C4- *Winslowiella sp.*, C5- *Brevundimonas diminuta*, C6-

*Staphylococcus sp.*, T2- *Bacillus sp.*, C3- *Bacillus licheniformis* and P2- *Klebsiella oxytoca.* These isolates may play a pivotal role in carbohydrate metabolism within the silkworm's digestive system, with C4 -*Winslowiella sp.* standing out as the highest α-amylase producer with activity 1.634±0.006 U/ml.

Similarly, the presence of cellulase enzyme producing gut bacteria in Eri silkworm was also reported by MsangoSoko *et al*. (2020b). Similarly, Prasanna *et al*. (2014) also characterized the presence of amylase producing bacteria in *B. mori*. However, the variation in the number of isolates and the enzymatic activity with the present work might be the differences in host organism, diet and environmental factors which resulted in changes (Yun *et al.,* 2014). This finding implied an adaptive response to the carbohydrate composition of the respective food plants associated with these isolates. Cellulase activity displayed substantial variation, with C5 (*Brevundimonas diminuta*) showcasing the highest levels with an activity level of 0.104±0.038 U/ml. This may suggests that certain gut bacteria, particularly *Brevundimonas sp*., possess a heightened ability to break down cellulose, potentially adapting to the fibre content of specific food plants. Similar finding was reported by Hu *et al.* (2014) who identified cellulose activity in the gut of *Dendroctonus armandi* larvae for the first time. However, there is no existing report on the enzymatic activity of *Brevundimonas diminuta* in Eri silkworm larvae. The diversity in cellulase activities reflects the adaptability of gut bacteria to the structural components of the ingested diet.

Proteinase enzymes play a crucial role in the breakdown of complex proteins into simpler forms. The detection of protease activity in gut bacterial isolates from the Eri silkworm affirmed the essential contribution of gut bacteria to the digestive processes of these insects. Recorded variations in proteinase activities among isolates could be indicative of their roles in breaking down proteins from diverse food sources.

Lipase activity, crucial for lipid digestion, was highest in isolates T1 (0.234±0.007 U/ml) and lowest in P4 (0.103±0.007) and interestingly both of them identified as *Mammaliicoccus sp*. This indicated the potential significance of these bacteria in breaking down and assimilating lipids from the silkworm's diet. Similarly, Feng *et al.* (2011) also found to have significant lipolytic activity in *B. mori* gut bacterial isolates. The observed variations in lipase activities emphasize the specificity of gut bacteria in responding to the lipid content of the respective food plants. The variations in lipolytic activity may possibly from the host organisms' ability to adapt to shifts in lipid quality, with the digestion of lipids being facilitated by a range of distinct lipase enzymes. Interestingly, some isolates exhibited lower overall digestive enzyme activities. For instance, isolates C1 and C2 which identified as genus *Mammaliicoccus,* displayed relatively lower cellulase activities, and P4 which was also a same strain *M. sciuri* showed reduced lipase activity might indicate that the activity pattern of gut bacterial isolates was dependent to diet of host organism. While these isolates may not be specialized for specific enzymatic activities, they may play complementary roles in the overall digestive process or occupy different niches within the silkworm's gut ecosystem. The observed variations in enzyme activities suggest a potential correlation between gut bacteria and the nutritional composition of the ingested plants. Certain bacterial species may be more adept at extracting nutrients from specific food sources, indicating a dynamic symbiotic relationship between gut bacteria and their host.

Among the four digestive enzyme activities studied ($\alpha$-amylase, cellulase, lipase, and proteinase), the maximum isolates exhibiting substantial roles were observed in the $\alpha$-amylase, cellulase and lipase activities category with highest from Sample C compare to Samples T and P, suggesting a widespread involvement of gut bacteria in carbohydrate metabolism and lipid metabolism within the Eri silkworm's digestive system. This finding of maximum bacterial isolates having ability to produce $\alpha$-amylase and cellulase aligned with the result of functional annotation where recoded maximum percentage of bacterial genome involvement in the carbohydrate metabolism pathway indicates a prevalent and crucial role for breakdown of complex carbohydrates, which were likely present in the silkworm's diet, which might helped larvae in hasten the digestion of carbohydrate. The bacterial isolates having role in this digestive enzyme activity belongs mainly to the class *Gamma-proteobacteria* and *Bacilli*, and belongs almost to the *Enterobacterales*.

Present study revealed a remarkable presence of class *Gamma-proteobacteria* and *Bacilli*, both constituting a dominant percentage. The prevalence of *Enterobacterales* in enzyme-producing bacterial isolates aligns with the findings of Gandotra *et al.* (2018a), emphasizing their significant role in amylase, cellulase, and lipase activity. This highlight the crucial contribution of bacterial isolates belonging to the order *Enterobacterales* in the digestion and nutrient absorption processes of lepidopteran insects. The genus *klebsiella*, identified as a prominent *Enterobacteriaceae* in this study, had been recognized as a beneficial microbe widely distributed in the gut of herbivorous insects. Previous reports by Suen *et al.* (2010), Chu *et al.* (2013); Acevedo *et al.* (2017); Yuan *et al.* (2021) emphasized its role in enhancing host fitness by promoting development, providing defence mechanisms, and improving insect survival and adaptability. Moreover, *Klebsiella oxytoca* had been implicated in augmenting food consumption and elevating haemolymph sugar and amino acid levels in *B. dorsalis* flies (Cai *et al*., 2018). Additionally, the enzyme-producing abilities of *Bacillus sp.* and *Citrobacter sp.* in silkworms had been consistently reported by various researchers, including Ramesh *et al.* (2009), Anand *et al.* (2010), Feng *et al.* (2011), Bhuyan *et al.* (2014), Prasanna *et al.* (2014), Bhuyan *et al.* (2018), Gandotra *et al.* (2018a), MsangoSoko *et al.* (2020a and b), Pandiarajan and Revathy (2020), MsangoSoko *et al.* (2021), Yuan *et al.* (2021), MsangoSoko *et al.* (2022).

The presence of the bacterial isolate *Mammaliicoccus sp.* in the gut of *S. ricini* across all three samples suggested a potential symbiotic role of *M. sciuri* in *S. ricini*. This correlation aligned with the findings of Douglas (1992), indicating that the gut bacterial flora in insects contributes metabolic functions not inherently present in the host insects. This microbial population's existence enhanced insect survival by improving digestion efficiency and supplying essential digestive enzymes and vitamins (Dillon and Dillon, 2004). While there was no prior evidence on the overall digestive enzyme activity of *M. sciuri* in lepidopteran insects, its prebiotic ability had been documented in goat milk by Naqqash *et al.* (2022). Overall, these findings illuminated the intricate digestive landscape within the Eri silkworm's gut, demonstrating the adaptability of gut bacteria to different dietary components.

The variations in enzyme activities among isolates indicated the diverse roles these microorganisms play in the digestion of carbohydrates, lipids, and proteins. This understanding contributed to the broader comprehension of insect-microbe interactions and their impact on the host's nutritional utilization, with implications for optimizing silkworm rearing conditions and potentially influencing silk production or other applications in the realm of insect biology.

The collective evidence from these studies may reinforce and extends our understanding of the intricate interplay between host plants, diet, and gut microbial dynamics. The consistent patterns observed across studies support the validity and broader relevance of the results, highlighting the significant influence of food plants on microbial diversity within the gut environment.