METAGENOMIC ANALYSIS OF ENDOSYMBIOTIC GUT BACTERIA ON VITAMIN C AUGMENTATION IN SILKWORM (*BOMBYX MORI* L.) UNDER THERMAL STRESS

A thesis submitted to the University of Calicut in partial fullfillment of the requirements for the award of the Degree of

DOCTOR OF PHILOSOPHY IN ZOOLOGY

Under the Faculty of Sciences

By

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Ph. D. Thesis in Zoology

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CERTIFICATE

This is to certify the thesis entitled "**Metagenomic Analysis of Endosymbiotic Gut Bacteria on Vitamin C Augmentation in Silkworm (*Bombyx mori.* L) under Thermal stress**" submitted to the University of Calicut by Mrs. Shahila Ismail. K. I. in partial fulfillment for the award of the Degree of Doctor of Philosophy in Zoology is a bonafide record of the research work carried out by her under the guidance of Dr. C. V. Sreeranjit Kumar, Professor (Retd.), Post Graduate and Research Department of Zoology, Govt. Victoria College, Palakkad.

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This is to certify that all the corrections/ suggestions from the adjudicators have been incorporated in the thesis entitled "**Metagenomic Analysis of Endosymbiotic Gut Bacteria on Vitamin C Augmentation in Silkworm (*Bombyx mori* L.) under Thermal Stress"** and the corrected copy is submitted to the University of Calicut for the award of the degree of Philosophy in Zoology and that the contents in the thesis and the soft copy are one and the same.

Place: **Dr. C. V. Sreeranjitkumar**

Date: (Research Guide)

DECLARATION

I, hereby declare that the work presented in the thesis entitled "**Metagenomic Analysis of Endosymbiotic Gut Bacteria on Vitamin C Augmentation in Silkworm (*Bombyx mori* L.) under Thermal Stress"** is based on the original work done by me under the guidance and supervision of Dr. C. V. Sreeranjit Kumar and has not been included in any other thesis submitted previously for the award of any degree. the contents of the thesis are undergone plagiarism check using iThenticate software at C.H.M.K. Library, University of Calicut, and the similarity index found within the permissible limit. I also declare that the thesis is free from AI generated contents.

Shahila Ismail K. I.

Signature of the supervising teacher

Place:

Date:

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Shahila Ismail K. I.

This research is dedicated to my Little Princess

Hazel Beema A. R.

And also, to

My lovely Family

For their everlasting Love, Affection, Support and

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CONTENTS

Preface

As insects constitute the largest animal group on the planet, there is a growing scientific interest in understanding their responses to environmental stresses, given their potential to medical, economic, and ecological benefits. One of the insects of significant economic importance is the silkworm (*Bombyx mori*), which is sensitive to its surroundings and susceptible to oxidative damage at elevated temperatures. To achieve a successful bivoltine silkworm crop, it is essential to rear the larvae under optimal conditions of temperature and humidity, providing them with the adequate quantity of good quality mulberry leaf. In this context, study has been conducted on silkworm larvae subjected to a diet supplemented with vitamin C under thermal stress. The objective was to investigate how these factors influence various aspects, including morphometric characteristics, gut symbionts, digestive enzymes, biochemical estimation, molecular parameters, and economic parameters related to cocoon production. The present studies are summarized as follows.

Chapter 1 Introduction:

This chapter gives a general introduction to the topic which includes thirteen subtopics. It covers the history of sericulture, importance of silk and role of gut symbionts. It describes the effect of thermal stress on the silkworm gut symbionts and associated digestive enzymes and also covers the importance of heat shock proteins. This also substantiates the role of nutrition and the importance of ascorbic acid in silkworm.

Chapter 2 Review of Literature:

This chapter gives a general review of the topic which is divided into eight subtopics. This chapter is devoted to recent findings on the effect of thermal stress on the gut bacteria and cocoon production in silkworms. It gives of the findings of the effect of the supplementation of vitamin C on silkworms.

Chapter 3 Materials and Methods:

This chapter describes the materials and methodology adopted to test the hypothesis. The experimental design and description of the sample preparations as well as the procedure adopted to investigate the gut bacterial characterisation, morphometric parameters of silkworm larvae, biochemical analysis, alterations in

the gut tissue, alterations in the expression of HSP genes and economic parameters of cocoon of silkworm exposed to thermal stress was presented.

Chapter 4 Results:

This chapter deals with the observations and results obtained in the present study. The results of the effect of supplementation of vitamin C on the gut symbionts, morphometric, biochemical, gene expression levels, alterations in the gut tissue and economic performance of silkworm on exposure to thermal stress were presented in this chapter.

Chapter 5 Discussion:

This chapter includes the discussion and interpretation of the data regarding the objectives of the experiment. The results obtained in the present study were related to previous studies and discussed their implications.

Chapter 6 Summary:

Summarizes the general conclusions from this research.

Chapter 7 Recommendations:

This chapter suggests Recommendations for future study.

Reference:

Gives the references used for the present study.

Abstract

The silkworm, *Bombyx mori* L. is a commercially important insect species that exhibit great temperature sensitivity and exhibit severe physiological and biochemical effects in the larval stage. Global warming is one of the serious threats that adversely affects the development and reproduction of silkworms. The optimum temperature for the normal growth and metabolism of silkworm is 20°C to 30°C and above that affects their regular development. Elevated temperatures have the potential to damage gut tissues and may invite oxidative destruction, resulting in a decrease in both the abundance of gut bacteria and the production of digestive enzymes. These enzymes play a crucial role in nutrient absorption, influencing the overall development of silkworms and the production of cocoons. The growth, development, and environmental adaptation of the host insect are greatly influenced by the gut symbionts. Numerous bacteria that support metabolic activities are found in their gut; however, very few studies have been reported about the diversity of these bacteria and their role in the growth and development of the silkworm. The present study compares the diversity of gut bacterial communities of silkworm under thermal stress and on supplementation of vitamin C. In order to assess the impact of vitamin C under high temperature 16S rRNA gene amplicon metagenomic analysis was conducted in the gut of fifth instar larvae. The morphometric parameters of silkworm larvae, alterations in the gut tissue, activity of digestive enzymes, mRNA expression levels of heat shock proteins and economic performance of cocoons were also studied for analysing the role of endosymbiotic bacteria in the development of the larvae and cocoon formation.

Fifth instar larvae were divided into four groups in order to compare the diversity of bacteria in their guts. One group was kept as control and fed with fresh mulberry leaves alone; the second group was fed with fresh mulberry leaves and subjected to a heat stress (40±2ºC) for an hour per day throughout the experimental period; the third group was fed with fresh mulberry leaves soaked in a 0.2% vitamin C solution; and the fourth group was exposed to both temperature and 0.2% vitamin C supplementation. The healthy fifth instar larvae were randomly sampled from each group and guts were dissected out in sterile condition before being homogenised and centrifuged for metagenomic analysis. The study used high throughput sequencing to

evaluate the impact of gut microbes of silkworms in response to high temperature and vitamin C supplementation.

The findings demonstrated that elevated temperature has a negative impact on the intestinal microbes of silkworm compared to the control and vitamin C supplemented group which were reared under optimum temperature (25 ± 3° C). Firmicutes, Proteobacteria and Bacteroidetes were the common bacteria found in all the four groups. The digestive process of silkworms may also be impaired by heat shock due to their effect on digestive enzymes. In comparison to thermal stress group the ascorbic acid-supplemented group were shown to have a higher level of enzyme activity. The activity of digestive enzymes showed a significant reduction in the thermal stress exposed group compared to the other experimental groups. Moreover, it was observed that the group treated with heat shock and supplemented with ascorbic acid showed a significantly increased activity than the group exposed to thermal stress. The length and weight of silkworm larvae and cocoons also showed a significant decrease when exposed to higher temperature but showed an elevation in the vitamin C supplemented group. The impact of high temperature also altered the gut architecture, and these changes may be detrimental to the normal functioning of the gut. However, the use of vitamin C is proposed as a potential solution to counteract the damages caused by the high temperatures. Vitamin C is known for its antioxidant properties, and it is suggested to have the capability to mitigate the negative effects of environmental stressors, such as high temperatures, on the gut architecture.

So, the results indicated that heat shock has an impact on the intestinal microflora of silkworms that control the activity of associated digestive enzymes which affects the digestion and nutritional intake, eventually impacting the growth and development of silkworm larvae and cocoons produced. These observations showed that the supplementation of vitamin C has the potential to counteract the stress brought on by heat shock even when subjected to high temperature. Moreover, vitamin C has the ability for enhancing the economic characteristics of silk as well as for the growth and development of silkworms by altering the gut bacterial diversity.

സംഗ്രഹം

പട്ടുനൂൽപ്പുഴു, *Bombyx mori* L., (Lepidoptera) വാണിജ്യപരമായി പ്രാധാന്യമുള്ള ഒരു പ്രാണിയാണ്, അത് വലിയ താപനില സംവേദനക്ഷമത പ്രകടിപ്പിക്കുകയും ലാർവ ഘട്ടത്തിൽ കഠിനമായ ശാരീരികവും ജൈവ രാസപരവുമായ പ്രത്യാഘാതങ്ങൾ പ്രകടിപ്പിക്കുകയും ചെയ്യുന്നു. പട്ടുനൂൽപ്പുഴുക്കളുടെ വളർച്ചയെയും പുനരുൽപാദനത്തെയും പ്രതികൂലമായി ബാധിക്കുന്ന ഗുരുതരമായ ഭീഷണികളിലൊന്നാണ് ആഗോളതാപനം. പട്ടുനൂൽപ്പുഴുവിന്റെ സാധാരണ വളർച്ചയ്ക്കും ജൈവരാസപ്രവർത്തനങ്ങൾക്കും ഏറ്റവും അനുയോജ്യമായ താപനില 20°C മുതൽ 30°C വരെയും അതിനു മുകളിലുള്ളതും അവയുടെ ക്രമമായ വളർച്ചയെ ബാധിക്കുന്നു. ഉയർന്ന ഊഷ്മാവ് കുടൽ കലകൾക്ക് കേടുപാടുകൾ വരുത്തുകയും ഓക്സിഡേറ്റീവ് നാശത്തെ ക്ഷണിച്ചുവരുത്തുകയും ചെയ്യും, ഇത് മൂലം കുടൽ ബാക്ടീരിയകളുടെ സമൃദ്ധിയും ദഹന രാസാഗ്നികളുടെ ഉത്പാദനവും കുറയുന്നു. ഈ രാസാഗ്നികൾ പോഷകങ്ങൾ ആഗിരണം ചെയ്യുന്നതിൽ നിർണായക പങ്ക് വഹിക്കുന്നു, പട്ടുനൂൽപ്പുഴുക്കളുടെ മൊത്തത്തിലുള്ള വളർച്ചയെയും കൊക്കൂണുകളുടെ ഉൽപാദനത്തെയും സ്വാധീനിക്കുന്നു. ആതിഥേയ പ്രാണികളുടെ വളർച്ച, വികസനം, പാരിസ്ഥിതിക പൊരുത്തപ്പെടുത്തൽ എന്നിവ ആന്തര സഹജീവികളെ വളരെയധികം സ്വാധീനിക്കുന്നു. ഉപാപചയ പ്രവർത്തനങ്ങളെ പിന്തുണയ്ക്കുന്ന നിരവധി ബാക്ടീരിയകൾ അവയുടെ കുടലിൽ കാണപ്പെടുന്നു; എന്നിരുന്നാലും, ഈ ബാക്ടീരിയകളുടെ വൈവിധ്യത്തെക്കുറിച്ചും പട്ടുനൂൽപ്പുഴുവിന്റെ വളർച്ചയിലും വികാസത്തിലും അവയുടെ പങ്കിനെക്കുറിച്ചും ഉള്ള പഠനങ്ങൾ വിരളമാണ്. ഇപ്പോഴത്തെ പഠനം, താപ സമ്മർദ്ദത്തിലും വൈറ്റമിൻ സി ചേരുവ കലർത്തി നൽകി പട്ടുനൂൽപ്പുഴുവിന്റെ കുടൽ ബാക്ടീരിയ സമൂഹങ്ങളുടെ വൈവിധ്യത്തെ താരതമ്യം ചെയ്യുന്നു. ഉയർന്ന താപനിലയിൽ വിറ്റാമിൻ സിയുടെ ആഘാതം വിലയിരുത്തുന്നതിനായി 16S rRNA ജീൻ ആംപ്ലിക്കൺ മെറ്റാജെനോമിക് വിശകലനം അഞ്ചാം ഘട്ട ലാർവകളുടെ കുടലിൽ നടത്തി. പട്ടുനൂൽ പുഴു ലാർവകളുടെ ബാഹ്യ ഘടനാ സവിശേഷതകൾ, കുടൽ കോശങ്ങളിലെ മാറ്റങ്ങൾ, ദഹന എൻസൈമുകളുടെ പ്രവർത്തനം, ഹീറ്റ് ഷോക്ക് പ്രോട്ടീനുകളുടെ mRNA എക്സ്പ്രഷൻ ലെവലുകൾ, കൊക്കൂണുകളുടെ

സാമ്പത്തിക മൂല്യം എന്നിവയും വികസന രീതി വിശകലനം ചെയ്യുന്നതിനായി പഠിച്ചു.

കുടലിലെ ബാക്ടീരിയകളുടെ വൈവിധ്യം താരതമ്യം ചെയ്യുന്നതിനായി അഞ്ചാം ഘട്ട ലാർവകളെ നാല് ഗ്രൂപ്പുകളായി തിരിച്ചിരിക്കുന്നു. ഒരു ഗ്രൂപ്പിനെ നിയന്ത്രണവിഭാഗമായി നിലനിർത്തുകയും ഇവയ്ക്ക് മൾബറി ഇലകൾ മാത്രം നൽകുകയും ചെയ്തു; രണ്ടാമത്തെ ഗ്രൂപ്പിന് മൾബറി ഇലകൾ നൽകുകയും പരീക്ഷണ കാലയളവിൽ ഓരോ ദിവസവും ഒരു മണിക്കൂർ താപ സമ്മർദ്ദത്തിന് (40± 2ºC) വിധേയമാക്കുകയും ചെയ്തു; മൂന്നാമത്തെ ഗ്രൂപ്പിന് 0.2% വിറ്റാമിൻ സി ലായനിയിൽ നനച്ച പുതിയ മൾബറി ഇലകൾ നൽകി; നാലാമത്തെ ഗ്രൂപ്പിന് താപസമ്മർദ്ദവും 0.2% വിറ്റാമിൻ സി ചേരുവയും നൽകി. ആരോഗ്യമുള്ള അഞ്ചാം ഘട്ട ലാർവകളെ ഓരോ ഗ്രൂപ്പിൽ നിന്നും ക്രമരഹിതമായി തിരഞ്ഞെടുക്കുകയും മെറ്റാജെനോമിക് വിശകലനത്തിനായി ഏകതാനമാക്കുകയും സെൻട്രിഫ്യൂജ് ചെയ്യുകയും ചെയ്യുന്നതിനുമുമ്പ് അണുവിമുക്തമായ അവസ്ഥ ഉറപ്പു വരുത്തി. ഉയർന്ന ഊഷ്മാവിനും വിറ്റാമിൻ സി സപ്ലിമെന്റേഷനും പ്രതികരണമായി പട്ടുനൂൽപ്പുഴുക്കളുടെ കുടൽ സൂക്ഷ്മാണുക്കളുടെ ആഘാതം വിലയിരുത്താൻ പഠനം മെറ്റാജെനോമിക് സീകൻസിങ് ഉപയോഗിച്ചു.

ശരാശരി ഊഷ്മാവിൽ (25 ± 3 ° C) വളർത്തുന്ന നിയന്ത്രണ വിഭാഗത്തെ വിറ്റാമിൻ സി സപ്ലിമെന്റഡ് ഗ്രൂപ്പുമായി താരതമ്യപ്പെടുത്തുമ്പോൾ ഉയർന്ന താപനില പട്ടുനൂൽപ്പുഴുവിന്റെ ആന്തര സൂക്ഷ്മാണുക്കളെ പ്രതികൂലമായി ബാധിക്കുമെന്ന് കണ്ടെത്തലുകൾ തെളിയിച്ചു. നാല് ഗ്രൂപ്പുകളായി കാണപ്പെടുന്ന സാധാരണ ബാക്ടീരിയകളാണ് ഫിർമിക്യൂട്ട്സ്, പ്രോട്ടിയോബാക്ടീരിയ, ബാക്ടീരിയോയിഡറ്റുകൾ. പട്ടുനൂൽപ്പുഴുക്കളുടെ ദഹനപ്രക്രിയയും ദഹന രാസാഗ്നികളെ സ്വാധീനിക്കുന്നതിനാൽ താപ സമ്മർദ്ദവും മൂലം തകരാറിലായതായ് കാണാൻ കഴിഞ്ഞു. തെർമൽ സ്ട്രെസ് ഗ്രൂപ്പുമായി താരതമ്യപ്പെടുത്തുമ്പോൾ, അസ്കോർബിക് ആസിഡ്-സപ്ലിമെന്റഡ് ഗ്രൂപ്പിന് ഉയർന്ന എൻസൈം പ്രവർത്തനം ഉണ്ടെന്ന് കാണിക്കുന്നു. മറ്റ് ചികിത്സാ ഗ്രൂപ്പുകളെ അപേക്ഷിച്ച് ദഹന എൻസൈമുകളുടെ പ്രവർത്തനം താപ സമ്മർദ്ദം വെളിപ്പെടുത്തുന്ന ഗ്രൂപ്പിൽ ഗണ്യമായ കുറവ് കാണിച്ചു. മാത്രമല്ല, താപസമ്മർദം ഉപയോഗിച്ച് ചികിത്സിക്കുകയും അസ്കോർബിക് ആസിഡുമായി സപ്ലിമെന്റ് ചെയ്യുകയും ചെയ്ത ഗ്രൂപ്പ് താപ സമ്മർദ്ദത്തിന് വിധേയരായ ഗ്രൂപ്പിനേക്കാൾ ഗണ്യമായ വർദ്ധനവ് കാണിക്കുന്നതായി

നിരീക്ഷിക്കപ്പെട്ടു. പട്ടുനൂൽ പുഴുക്കളുടെയും കൊക്കൂണുകളുടെയും നീളവും ഭാരവും ഉയർന്ന താപനിലയിൽ സമ്പർക്കം പുലർത്തുമ്പോൾ ഗണ്യമായ കുറവ് കാണിച്ചുവെങ്കിലും വിറ്റാമിൻ സി സപ്ലിമെന്റഡ് ഗ്രൂപ്പിൽ ഉയർച്ച കാണിച്ചു. ഉയർന്ന താപനിലയുടെ ആഘാതം കുടലിന്റെ വാസ്തുവിദ്യയിലും മാറ്റം വരുത്തി, ഈ മാറ്റങ്ങൾ കുടലിന്റെ സാധാരണ പ്രവർത്തനത്തിന് ഹാനികരമായേക്കാം. എന്നിരുന്നാലും, ഉയർന്ന താപനില മൂലമുണ്ടാകുന്ന നാശനഷ്ടങ്ങളെ പ്രതിരോധിക്കാനുള്ള സാധ്യതയുള്ള പരിഹാരമായി വിറ്റാമിൻ സിയുടെ ഉപയോഗം നിർദ്ദേശിക്കപ്പെടുന്നു. വിറ്റാമിൻ സി അതിന്റെ ആന്റിഓക്സിഡന്റ് ഗുണങ്ങൾക്ക് പേരുകേട്ടതാണ്, കൂടാതെ കുടൽ ഘടന ഉയർന്ന താപനില പോലുള്ള പാരിസ്ഥിതിക സമ്മർദ്ദങ്ങളുടെ പ്രതികൂല ഫലങ്ങൾ ലഘൂകരിക്കാനുള്ള കഴിവ് ഇതിന് ഉണ്ടെന്ന് നിർദ്ദേശിക്കപ്പെടുന്നു.

അതിനാൽ, താപസമ്മർദം പട്ടുനൂൽ പുഴുക്കളുടെ കുടൽ മൈക്രോഫ്ലോറയിൽ സ്വാധീനം ചെലുത്തുന്നു, ഇത് ദഹനത്തെയും പോഷകാഹാരത്തെയും ബാധിക്കുന്ന അനുബന്ധ ദഹന രാസാഗ്നികളുടെ പ്രവർത്തനത്തെ നിയന്ത്രിക്കുന്നു, ഇത് ഒടുവിൽ പട്ടുനൂൽ പുഴുക്കളുടെയും കൊക്കൂണുകളുടെയും വളർച്ചയെയും വികാസത്തെയും ബാധിക്കുന്നു. ഉയർന്ന ഊഷ്മാവിന് വിധേയമാകുമ്പോഴും താപസമ്മർദം മൂലമുണ്ടാകുന്ന ആഘാതത്തെ പ്രതിരോധിക്കാൻ വിറ്റാമിൻ സിയുടെ സപ്ലിമെന്റിന് കഴിവുണ്ടെന്ന് ഈ നിരീക്ഷണങ്ങൾ കാണിച്ചു. കൂടാതെ, വൈറ്റമിൻ സിക്ക് പട്ടിന്റെ സാമ്പത്തിക മൂല്യം വർദ്ധിപ്പിക്കാനും ആന്തരിക ബാക്ടീരിയ വൈവിധ്യത്തിൽ മാറ്റം വരുത്തി പട്ടുനൂൽപ്പുഴുക്കളുടെ വളർച്ചയ്ക്കും വികാസത്തിനും കഴിവുണ്ട്.

List of Abbreviations

B. mori	-	*Bombyx mori*
BmCPV	-	*B. mori* cypovirus infection
BmHSP	-	*Bombyx mori* Heat Shock Protein
CN	-	Control
HSP	-	Heat Shock Protein
MT	-	Million Tons
NGS	-	Next-generation sequencing
OUT	-	Operation Taxonomic Units
QIIME	-	Quantitative Insights into Microbial Ecology
T1	-	Thermal stress exposed group
T2	-	Vitamin C supplemented group
T3	-	Vitamin C supplemented + Thermal stress exposed group
THTT	-	Transient high temperature treatment

List of Publications

1. Peer Reviewed International Journal Paper

i. Shahila Ismail K. I., Sreeranjit Kumar C. V., Aneesha, U., Syama, P. S., & Sajini, K. P. (2023). Comparative analysis of gut bacteria of silkworm *Bombyx mori* L. on exposure to temperature through 16S rRNA high throughput metagenomic sequencing. *Journal of Invertebrate Pathology*, *201*, 107992. DOI:10.1016/j.jip.2023.107992

2. List of Papers/ Posters presented at conferences

2.1. International Conference

i. Shahila Ismail K. I., Sreeranjit Kumar C. V. Impact of vitamin C supplementation on the gut bacteria of silkworm *Bombyx mori* L. under thermal stress through metagenomic analysis. International Conference on recent Advances in Animal Biology and Conservation (ICRABC 2023) held at University College, Thiruvananthapuram, Kerala, India. December 19th – 21st 2023.

2.2. National Conferences

i. Shahila Ismail K. I., Sreeranjit Kumar C. V. Impact of ascorbic acid fortification in the activity of digestive enzymes and cocoon production of silkworm *Bombyx mori* L. under thermal stress. Three Day National Seminar on Biodiversity for Sustainable Future held at Sree Neelakanda Govt. Sanskrit College, Pattambi, Palakkad, Kerala, India. November 14th to 16th 2023.

ii. Shahila Ismail K. I., Sreeranjit Kumar C. V. The activity of digestive enzymes and cocoon production of silkworm *Bombyx mori* L. on supplementation of ascorbic acid under thermal stress. National Seminar on Intersections: Advances in Interdisciplinary Research held at St. Thomas College, Thrissur, Kerala, India. February 24- 25 2023.

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CHAPTER 1

INTRODUCTION

1. Introduction

1.1. **Sericulture- an overview**

Sericulture is the scientific practice dedicated to the cultivation of silkworms for silk production. The term "sericulture" finds its roots in the Latin word "Serio," meaning silk. This agro-based enterprise revolves around the meticulous rearing of silkworms to yield the highly coveted and economically significant product, silk. Renowned as the "queen of textiles" due to its exquisite, resilient, lustrous texture, soft touch, long-lasting quality, and durability, silk holds a special place in the world of textiles (The World Book Encyclopedia, Silk, 1992). China was said to have been the origin of silk production as early as the Neolithic era. According to mythology, silk was found in China between 2600 and 2700 B.C., during the reign of the great emperor Haung Ti. It then gradually travelled east and west over the so-called Silk Road. Silkworm rearing has been done in China from 2500 BC, according to biological and archaeological data (Kuhn, 1988). The hypothesis that the domesticated silk moth, *Bombyx mori*, developed from its wild progenitor, *Bombyx mandarina*, about 4600 years ago is supported by the fact that the oldest piece of silk fabric known to science goes back to 5000 years (Hirobe, 1968). Genetic hybridization was introduced in the early Taisho era (1912–1926), leading to the development of 1250 variants. Over the subsequent Taisho–Showa era, this number significantly increased, reaching a total of 2000 varieties by the end of that period (Hiratsuka, 1969). In nations like Brazil, China, France, India, Italy, Japan, Korea, and Russia, sericulture has developed to be a significant cottage industry. China and India are the two leading producers nowadays with over 60% of global output annually (Collard *et al*., 2005).

1.2. **Sericulture status in India**

Indian culture and way of life have seamlessly intertwined with the essence of silk. Although the precise origin of sericulture in India remains uncertain, its roots extend far into antiquity. India boasts a rich and sophisticated history of silk production, marked by a silk trade that can be traced back to the fourteenth century. The art of sericulture has become an integral part of India's cultural tapestry, contributing to its historical and artistic legacy. In India, the global sericulture industry employs over 8.25 million people in rural and semi-urban regions. India is the world's largest consumer of silk and silk textiles as well as the world's second-largest producer of

raw silk, behind China. Tamil Nadu, Kerala, Karnataka, West Bengal, Andhra Pradesh, Jammu and Kashmir, Gujarat, Bihar, Maharashtra, Orissa, Rajasthan, and Uttar Pradesh are among the states where sericulture is prevalent (Gurjar *et al*., 2018). In India, sericulture is a labour- intensive, farm-based industry that benefits rural farmers by offering high employment and profitable returns. Women do around 53% of the activities related to sericulture. India holds a special position in the global economy since it produces the four commercial kinds of silk: mulberry, tasar, muga, and eri. Only Assam produces the muga silk, which is golden yellow. Of the 28,472 million tons of raw silk produced in 2015–16, Mulberry accounted for 71.8% (20,434 MT), Tasar for 9.9% (2,818 MT), Eri for 17.8% (5,054 MT), and Muga for 0.6% (166 MT) (Sai and Ali, 2021)

Combining agriculture and industry, sericulture is one of the labour-intensive sectors of the Indian economy, giving a significant portion of the population a means of livelihood in employment opportunities. In the realm of agriculture, it stands out as the sole cash crop capable of delivering results in under 30 days. Almost three and a half million individuals in our nation are employed in this business. Given its high employment potential, low capital intensity, and profitable production, it serves as a livelihood for millions of individuals. Notably, a substantial portion of the workforce engaged in this industry includes women and other economically disadvantaged groups. India has achieved the top position in the silk industry owing to its rich historical and cultural home market and enormous range of silk garments that accurately reflect regional uniqueness.

Enhancing the quality and productivity of silk is the primary goal for the Indian government. The Ministry of Textiles, Government of India, has ultimate administrative responsibility over the Central Silk Board, which is responsible for developing the silk sector. The government-owned Sericulture Departments work to promote the sericulture sector through marketing, seed supply, extension, and other means. Research and technology development for profit-making in sericulture is also carried out by state departments of sericulture and universities.

1.3. **Silkworm as an ideal model organism**

The silkworm *B. mori* L. is a phytophagous lepidopteran insect which exclusively forages on mulberry leaves (*Morus alba* L.). It is an important sericigenous insect

which helps in the production of silk protein by altering the mulberry leaf proteins (Babu *et al.*, 2009). *Bombyx mori* holds economic importance as it is utilized in silk production and serves as a valuable model organism for biological research. Due to their short generation period, distinct genetic background, abundance of genetic resources, silkworms have been extensively employed in a wide range of life science investigations (Tamura *et al*., 2000., Tomita *et al*., 2003). China and Japan began working on the silkworm genome project in 2003, and by the time it was finished, the silkworm had three draught maps, a fine map, and a multistrain genome re-sequencing (Xia *et al*., 2004, Xia *et al*. 2009). This tremendously accelerated the advancement of sericulture science and made the silkworm an excellent model organism for scientific study.

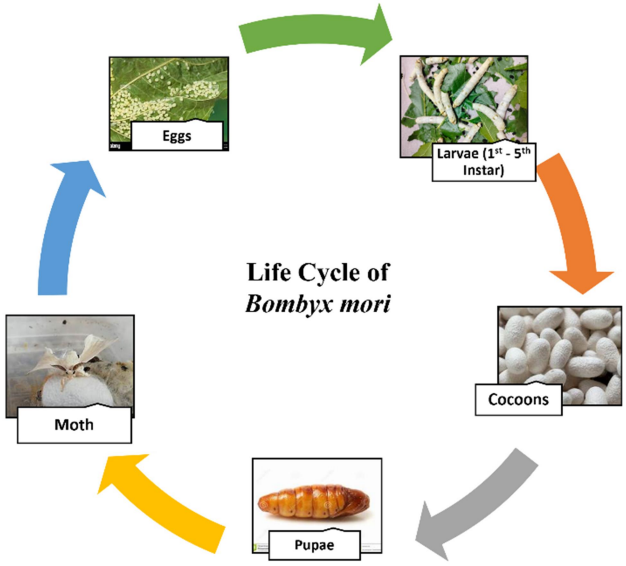
According to Nwibo *et al*. (2015), the use of silkworm models is being used for a variety of life science applications, including environmental safety monitoring, antipathogenic medication screening, and treatment assessment. Silkworms exhibit substantial progeny sizes and rapid generation turnover, completing a 25–30-day larval stage. Rich in genetic features, they possess 28 pairs of chromosomes, a notable contrast to *Drosophila melanogaster*, which has only four pairs. There are no bioethical concerns associated with its death, whether it is forced or genetic (Chen *et al*. 2014). Furthermore, the silkworm possesses a moderate body size, making it easy to dissect and extract various tissues and organs, including the hemolymph, fat body, midgut, and silk gland. Moreover, studies involving oral administration and intravenous injection that resemble those involving mammals may be conducted using silkworms. Selecting a model animal for environmental monitoring is essential to the evaluation of ecological environment safety. According to Sekimura (2005) and Hamamoto *et al*. (2009), silkworms are particularly vulnerable to pesticides, heavy metals, and other dangerous substances, as well as fluctuations in climate change. After the silkworm genome project was completed and a protein and genomic database was established, silkworms started to become recognised as useful models for scientific study (Mita, 2008).

1.4. **Life cycle of silkworm**

The silkworm *B. mori* is a holometabolous insect which completes its life cycle in 45 to 55 days and consists of 4 stages; egg, larvae, pupa and moth. The egg stage consists

of 9-10 days, larval stage lasts for 24 to 28 days which consist of 5 instars (feeding periods) and four moults. The initial three stages are known as chowki worms or young age worms and final two instars are referred to as old age worms. Larvae reach a maximum length of around 70 mm in the last and fifth instar, which is 8,000–10,000 times longer than they were in the first instar. The cocoon begins to spin as soon as it is mounted. In order to create a fine, long, and firm silk thread, the larvae release liquid silk from the spinneret during this period. The cocoon, or pupal shell, is formed by these strands spinning around the larvae. The larval structures go through metamorphosis throughout the 8–10-day pupal stage. After degenerating, the larvae transform into adult moths. The moth has scales covering its body and is tinted a creamy white. Their lifespan is three to ten days, and they are unable to fly or eat. Compared to male moths, females have a wider abdomen. Male moth will die soon after fertilization and the female moth dies after egg laying and the life cycle continues. More than 350 eggs were produced by female moths at a time.

Figure 1.1. Life Cycle of Silkworm *Bombyx mori* L.



1.5. **Types of silkworms**

India boasts the distinctive production of five economically significant varieties of natural silk, each originating from different types of silkworms that feed on various host plants. Apart from mulberry silk, collectively categorized as non-mulberry silks, these diverse types contribute to India's rich silk industry. This broad spectrum of silk

production stands as a unique and noteworthy feature in the country's commercial landscape

Bombyx mori L., a silkworm that only eats the leaves of mulberry plants, is the source of mulberry silk. These silkworms have been reared inside and have undergone total domestication. Tasar (Tussah) is a coarse silk with a coppery hue that is mostly utilised for interior design and furniture and it is the culture of Indian tribal communities. The silkworm *Antheraea mylitta*, which mostly feeds on the food plants, produces tasar silk and its rearing takes place outside on exposed trees. Oak Tasar, a more delicate form of tasar, is generated by *Antheraea proyeli* J. This silkworm thrives on oak trees, which serve as its natural food source in the sub-Himalayan region of India. Eri, often referred to as Endi or Errandi, is a multivoltine silk that, in contrast to other types of silk, is spun from open-ended cocoons. The farmed silkworm, *Philosamia ricini*, produces eri silk, which is primarily fed castor plants. Muga silk with a golden yellow hue is a national treasure and a source of pride for the state of Assam. *Antheraea assamensis*, a semi-domesticated multivoltine silkworm, is the source of it. These silkworms are raised on trees resembling tasars and fed on the fragrant leaves of Som and Soalu plants. (Giridhar *et al*., 2010).

Silkworm races are categorised based on the number of generations per year. A single generation is completed by the univoltine race annually. On the other hand, the bivoltine races finish two generations in a year, while the polyvoltine races finish six or seven generations. Univoltine and Bivoltine eggs typically lay pale yellow eggs that progressively become brown one to two days after they are deposited. The deep yellow eggs deposited by polyvoltine moths remain unchanged for a duration of one to two days. While bi-voltine races lay diapause eggs in the second generation after non-diapausing eggs in the first, univoltine breeds always lay diapausing eggs. Eggs from multivoltine breeds are never diapausing. On the other hand, voltinism may be artificially changed.

1.6. **Gut bacteria in silkworm**

The digestive tract of silkworm larvae is almost entirely composed of a single tube that runs from the mouth to the anus. This tube is classified as the fore-gut, or stomodeum, the mid-gut, or mesenteron, and the hind gut, or proctodeum. The

oesophagus is constricted in the front and is progressively wider in the back. The stomodeal is located at the end of the foregut, preventing backflow and retaining part of the contents. The gut is a long, broad, cylindrical tube that becomes smaller towards the posterior end. Food is mostly broken down and assimilated in the mid-gut. Food that has been broken down is absorbed by the cylindrical cells, and the goblet cells in the mid-gut epithelium are primarily responsible for secreting the digestive fluid. The epithelium of the midgut is always simple and made up of several cell types. The predominant cell type is the columnar cell. Columnar cells can take on a variety of shapes and functions, including those related to water absorption, secretion, digestion by enzymes linked to microvillar membranes, and nutrient absorption; regenerative cells are frequently grouped together at the base of the epithelium and specialised cells such as columnar and goblet cells (Chapman, 1998). Some of them are goblet cells, which actively move potassium ions from the hemolymph to the lumen of the midgut (Harvey *et al*., 1983).

One of the key internal environmental factors influencing the activity of digestive enzymes is the pH of the contents of the gut. Numerous studies have revealed the gut pH values of numerous insects as well as the related pH optima of their digestive enzymes. The conclusion drawn from these investigations was that the luminal pH in insect gut and enzyme pH maxima are correlated (Applebaum *et al*., 1985). Lepidoptera insects, whether they feed on leaves (the majority of species), wax (Galleria mellonella), or keratin (Tineola bisselliella), have extremely alkaline midgut contents, especially in the middle ventriculus. The progenitors of leaf-eating Lepidoptera may have adapted to this high pH in order to remove hemicelluloses from plant cell walls (Terra *et al*., 1988).

During the larval stage, the gut tissue carries out a crucial function in the processes of digestion, absorption, and nutrient transportation. The brush border membrane in the gut tissue increases the surface area of the intestinal cell membrane and this considerably improves the organism's efficiency for digestion and absorption (Javed *et al*., 2019). The growth and development of organisms heavily rely on the functions carried out by digestive enzymes. These enzymes are integral in breaking down and assimilating nutrients, ensuring that essential building blocks are available for various biological processes that contribute to overall growth and developmental milestones.

Protease, amylase, lipase and cellulase are the chief digestive enzymes present in the insect gut (Terra & Ferreira 1994).

Through appropriate food digestion and the use of food obtained from their surroundings, insects are able to meet their nutritional needs. Insects consume polymers, which are broken down into three stages of digestion, including proteins, lipids, and carbohydrates. The dispersion and molecular size reduction of the polymers that result in oligomers is known as primary digestion. A further reduction occurs during intermediate digestion, resulting in smaller molecules known as dimers. The ultimate phase involves the conversion of dimers into monomers. Specialized digestive enzymes, strategically positioned within the insect gut, meticulously oversee and control this conversion process with precision.

Protease, the protein digestive enzyme is responsible for the dietary protein metabolism which hydrolyses molecular proteins into peptides and amino acids (Horie *et. al*, 1982). During the larval stage, activity of protease enzymes is at a higher rate that may enhance the production of silk proteins from mulberry leaf (Eguchi *et al*., 1972). α-Amylases, also known as α-1,4-glucan-4-glucanohydrolases, are hydrolytic enzymes responsible for catalyzing the hydrolysis of α-D-(1,4)-glucan linkages present in glycogen and similar carbohydrates (Zibaee, 2012). Amylases are the key enzymes which are involved in the digestion of carbohydrates and the lipase perform an essential role in the digestion and processing of dietary lipids into triglycerides. Animals, plants, and microbes all have lipases, or triacylglycerol-acyl-hydrolase, which catalyses the hydrolysis of fatty acid ester linkages. The action of lipases on substrates at the aqueous-lipid phase interface is their most distinctive feature.

Cellulase catalyses the hydrolysis of cellulose present in the mulberry leaves into simple sugars. (Li *et al*., 2009a). Several microbes, mostly bacteria and fungi, generate this enzyme (Immanuel *et al*., 2006). Cellulase is a crucial enzyme that facilitates the depolymerization of cellulose into fermentable sugar (Xing *et al*., 2009). According to Lee and Koo (2001), cellulases are the bioactive compounds that microorganisms produce when they grow on cellulosic substrates. Microorganisms that break down cellulose are capable of doing so by using either an acid or an enzyme to hydrolyze it into soluble sugars. Certain bacterial species, such as

Micrococcus, Bacillus, Pseudomonas, and Cellulomonas, possess cellulolytic properties (Nakamura & Kitamura, 1982). Bacillus spp. is among the bacteria that generate a variety of extracellular enzymes, such as proteinases, amylases, and polysaccharide hydrolases (Mawadza *et al*., 2000)

Insects, the most diverse group of creatures on Earth, stand as one of the planet's oldest species. Found ubiquitously in nature, they demonstrate remarkable adaptability to a wide array of environmental conditions. (Chapman *et al*., 2009). Subsequently, a variety of bacteria have been linked to these insects, and the microorganisms that are colonising the insects may be harmful or symbiotic (Bode, 2011). The group of microorganisms that colonise the intestines of vertebrate and invertebrate species, including bacteria, archaebacteria, and eukarya, is referred to as the "gut microbiota" or "gut microflora." According to Beackhed *et al*. (2005), these bacteria have co-evolved with their host for over a million years, resulting in a mutualistic connection inside the developing host. As per Savage (1978), the extensive and diverse microbial communities residing in the digestive tracts of insects serve as yet another example of the remarkable diversity inherent in these creatures.

It is estimated that there are almost ten times as many bacteria in the gut of insects than the human gut (Rajagopal, 2009). It has been discovered that the gut microflora has more than 100 times the genetic material of the human genome (Gill *et al*., 2006). The host and its microbiota are referred to as "superorganisms" because of the quantity of bacterial cells in the body (Gill *et al*., 2006). According to Douglas (2015), an insect is a "multiorganismal entity" if its microbiota makes up between 1 and 10% of its total biomass. The digestive systems of insects offer distinct environments for microbial colonization, and among these microorganisms, gut bacteria play a crucial role in providing various beneficial functions to their hosts. The degree to which insects depend on gut microbes for essential processes can vary significantly. Insects get advantages from their resident bacteria through the production of vital chemicals, digestion and metabolism, vitamin absorption, resistance to infections, pheromone generation, immunity, and more (Dillon and Dillon, 2004; Rajagopal, 2009).

The gut bacteria play a critical role in the survival of insects by protecting their hosts from pathogens and natural enemies through the activation of the insects' immune

systems to combat the invader organism (Douglas, 2015). The microbiota of insects is crucial for their ability to adapt to different kinds of food and has been demonstrated to be significant for environmental adaptation, nutrient synthesis, and compound detoxification (Despress *et al*., 2007; Shi *et al*., 2011). Microbial analysis has been a prominent method for studying the composition, function, and development of diverse microbiota, from the deep sea to the human gut system (Reason *et al*., 2003). These factors have spurred a contemporary surge in comprehensive research on the microbiome of insects.

Alterations in gut microbial composition and activity are correlated with variations in gut tissue damage and metabolism in silkworms inhabiting tropical environments. (Ismail *et al.,* 2023). The gut bacteria have a significant impact on host physiology, spanning from immunology to gut structure and is essential for the development and environmental adaptation of the host insect (Tefit and Leulier, 2017; Qiao *et al*., 2019). Previous studies have demonstrated that both host and microbial genes work in concert to control the metabolic process (Broderick and Lemaiture, 2012; Dayama *et al*., 2020). In response to changes in the availability of nutrients and environmental factors, the composition as well as the diversity of the gut bacteria of silkworm will alter and have an impact on the production and quality of cocoons (Yuan *et al*., 2006).

The intestinal microflora secretes a large number of carbohydrates digesting enzymes for degrading carbohydrates which is used as the major source of energy for the host. (Xu *et al*., 2003; Krajmalnik *et al*., 2012). Gut microbes are also capable of breaking down proteins into amino acids which are transported by certain transporters into the bacterial cells (Davila *et al*., 2013). In addition, these substances have the ability to enter the metabolic pathways directly through the fermentation by several anaerobic bacteria, which leads to the production of organic acids, fatty acids and ethanol (Louis *et al*., 2007). Studies have demonstrated that gut bacteria can cause the accumulation of fat in the host by controlling the expression of the host's genes and by altering the host's energy metabolism (Turnbaugh *et al*., 2008; Buchon and Osman, 2015; Fan and Pedersen, 2021). The metabolic process of the host becomes aberrant and has an impact on the growth and development, if the balance between the intestinal bacteria and the environment of the host is compromised (Martin-Nuñez *et al*., 2021).

1.7. **Effect of temperature on silkworm**

For species like insects, whose physiological processes rely on external temperature regulation, adapting to shifting climatic circumstances is especially challenging. Since many insects lack the physiological tolerance necessary to withstand prolonged exposure to the current extreme temperatures found in their habitats, they are under grave threat from global warming. The frequency of heat waves and persistent high temperatures brought on by global warming affect the reproduction and survival rate of insects (Stazione *et al*., 2019; González-Tokman *et al*., 2020; Abdel-Hady *et al*., 2021). The silkworm is not equipped with a full body temperature regulation and maintenance system. The silkworm's body temperature is directly influenced and essentially determined by the temperature of its surroundings. The mulberry silkworm is extremely delicate and susceptible to changes in its surroundings, and unable to withstand drastic variations in humidity and temperature that occur naturally.

The success of sericulture is not only dependent on their nutrition, but also influenced by their environmental condition, especially temperature (Murugan and George, 1992). As silkworms are very sensitive due to artificial domestication and indoor upbringing, the fluctuations in the environmental temperature affects the sericulture industry severely (Sugnana *et al*., 2011; Jiang *et al*., 2021). It is generally known that genetic and economic characteristics, and disease resistance of silkworm are all closely correlated with the environmental temperature (Lee *et al*., 2005; Sugnana *et al*., 2011). The environment, encompassing factors such as temperature, humidity, and photoperiod, collectively influences the life cycle of the silkworm and, consequently, the production of cocoons. (Murugan and George, 1992).

Since silkworms are poikilothermic insects, one of the key abiotic elements influencing their development and production is temperature (Benchamin and Jolly, 1986). The physiology of the silkworms, including blood circulation, respiration, digestion, and nutritional absorption, is directly impacted by the temperature during rearing. Depending on the variety and developmental stage of the silkworm, different temperatures are optimal for its healthy growth. Elevated temperatures have an impact on the quality of mulberry leaves as well. The most appropriate temperature range for the growth and development of silkworm is 20 to 30° C (Khan, 2014). The temperature range between 35 and 45 °C is sublethal to the silkworm which is

characterised by negative impact in their development and it may result in a thermal coma or even fatal if they are exposed to high temperature for a prolonged duration (Xiang *et al*., 2005). Numerous studies have reported that high-quality cocoons are produced at temperatures between 22 and 27°C, and that any temperature above these ranges results in low-quality cocoons (Suresh and Harjeet, 2011). Temperatures exceeding 30°C have been found to have a direct impact on silkworm health (Devi and Karuna, 2012). All physiological processes slow down if the temperature falls below 20°C.

The fifth instar is the most active stage of larval development, during which time they store up a significant amount of food reserves that are then used for cocoon spinning, transformation, and reproduction (Hugar *et al*., 1998). Reports showed that the fourth and fifth instars of silkworms were more vulnerable to higher temperatures (Ueda and Lizuka, 1962; Shirota, 1992). High temperature shortens the development of fifth instar larvae which also impaired their ability to absorb and assimilate food (Rahmathulla and Suresh, 2012). High temperatures—particularly those above 30°C—do not promote larval development during the mating season and ultimately result in larval death (Hussain *et al*., 2011). According to Kremky and Michalska (2004), silk worm larvae spun their best cocoons at 25°C. The moulting period is significantly impacted by temperature changes as well as fluctuations in relative humidity (Mishra and Upadhyay, 2002). All developmental phases of the multivoltine race, strains C. Nicini of *B. mori*, may be fatally affected by temperatures exceeding 43° C (Omana and Karumathil, 1995).

High temperatures have the potential to influence the number or quality of cocoon crops generated by silkworms, and therefore, the amount of silk that is produced (Tazima and Ohuma, 1995). Increased temperatures adversely affect nearly every biological activity, resulting in the deterioration of biochemical and physiological responses. These, in turn, impact the quantity and quality of harvested cocoons. (Willmer *et al*., 2004., Siddiqui *et al*., 2005). Previous studies showed that exposure to high temperatures altered the protein production of posterior silk glands and even their gene expression profiles of silkworms. (Li *et al*., 2012; Li *et al*., 2014; Wang *et al*., 2014a). The usual pattern of protein synthesis decreased due to the temperature stress, which results in many anomalies at the cellular level (Feder, 1996). Studies

also revealed that high temperature can alter the composition and diversity of gut microflora of *B. mori* (Sun *et al*., 2017).

Late-stage larvae showed high tolerance levels when compared to adult moths and eggs. He also noted that exposure to 17° C and 33° C was similarly tolerated, but temperatures over 43°C proved to be fatal (Singh *et al*, 2013). Suresh and Harjeet (2011) have developed bivoltine double hybrids that are resistant to the high temperatures and high humidity of the tropics. The precise impact of climate change on sericulture depends on temperature, which could rise between 0.5 and 4.0° C in different parts of the nation over the course of the next few decades due to the buildup of anthropogenic greenhouse gases in the atmosphere. (Ram *et al*., 2016). This could have a negative impact on sericulture practices and the economy in temperate regions of India and a positive or marginal impact in tropical regions.

Table 1.1. The optimum temperature required for various larval stages of silkworm (Rahmathulla and Suresh., 2012).

Instars	Temperature range
1st Instar	27 - 28° C
2nd Instar	26 - 27° C
3rd Instar	26 - 27° C
4th Instar	24 - 25° C
5th Instar	23 - 23° C

The temperature zone of silkworm is classified into five categories based on the level of temperature exposure. **Lethal high temperature zone** (45–50 °C), in which the silkworms are first aroused by the high temperature and subsequently enter a coma. Certain proteins coagulate and the body's enzyme system is damaged. Silkworms perished quickly, and even if they relocated to an appropriate temperature range, they would not be able to bounce back. In **sub-lethal high temperature zones** (35–45 °C), an inappropriately high temperature throws the silkworm's assimilation and dissimilation out of balance, which shows up as stunted growth and development. In severe circumstances, it might result in death. If the duration is too lengthy, it can potentially produce thermal coma. You can still go back to normal if you relocate to a proper temperature zone after spending a little period of time in this one. The

optimum temperature zone (7~35 ℃) is the actual rearing temperature of silkworms in which the growth and development are adequate, and life activities are normal. The **sublethal low temperature zone** (7~ -10 ℃) is the state in which the metabolic intensity of silkworms drastically drops and enters into a coma stage. Serious instances cause bodily fluids to freeze. For a brief while at this temperature, silkworms can continue their activity after shifting to a place with an appropriate temperature; however, continuing too long can result in mortality. In **Lethal low temperature zone** (10~ -15℃), a large amount of the silkworm's bodily fluid freezes and crystallises, dehydrating the protoplasm as a result of the ice crystals' mechanical damage and destroying the physiological function structure, which ultimately results in the silkworm's death (www.pandasilk.com/the-influence-of-environmental-temperature-on-silkworm).

1.8 Heat Shock Proteins (Hsps)

Typically, insects require a variety of ambient temperatures in order to optimise their physiological and metabolic activities. The organisms start to suffer from heat stress when the temperature rises over the critical point (Neven, 2000). According to Jorgensen *et al*. (2006), insects that are under heat stress may exhibit physiological and metabolic anomalies that might hinder their ability to function normally. Most insects are capable of experiencing extremely high or low temperatures. Deadly high temperatures often fall between 40 to 50° C, depending on the kind of insect species and their individual developmental phases (Terblanche *et al*., 2011). An insect's behaviour and growth are greatly impacted by heat stress. This is reflected in developmental delays, sluggish development, lower reproduction, higher mortality, and inadequate embryo development (Zhang *et al*., 2014). Heat shock proteins, which act as molecular chaperons to protect proteins during their folding process, are produced by insects exposed to high temperatures. Heat shock proteins (Hsps) are not only sequentially expressed during the normal cell cycle development process, but they are also triggered in cells under a range of stress conditions caused by infections, tumours, temperature changes, cellular damage, and other variables. They are ubiquitous components that may be found in both plant and animal cells. In Drosophila salivary glands, they manifest as "chromosomal puffs" (Ritossa, 1962).

Heat shock proteins are produced more often in stressful situations that help in the capacity of cells to withstand thermal shock. However, this might have detrimental impacts on the physiology and developmental processes of organisms. For example, in order to avoid or minimise the effects of stress, insects have developed a variety of behavioural and physiological adaptations (Malik and Reddy, 2009). Their bodies generate Hsps and activate particular genes in response to such circumstances (Hong *et al*., 2010). Heat shock proteins are present in cells under normal conditions, but they express themselves massively in response to a sudden change in temperature (Zhao and Jones, 2012). As a result, the Hsps family is regarded as one of the most important and contentious areas of current study. According to Multhoff *et al*. (1998), these proteins are very abundant in a variety of cellular components, including the cytosol, nucleus, nucleolus, endoplasmic reticulum, mitochondria, lysosomes, and plasma membrane. They are evolutionarily conserved proteins. It is divided into many subgroups based on sequence homology, molecular weight, and function. This comprises Hsp40, Hsp70, Hsp90, Hsp100, and small Hsps (sHsps), among others.

Table 1.2. Major heat shock proteins and their roles (Smith *et al*., 1998).

Major Family	Cellular Localization	Cellular Function
Small heat shock proteins	Cytosol/ Nucleus	Protecting cells during stress and maintenance of a cytoskeletal structure.
Hsp40	Cytosol/ Nucleus/Mitochondria	Stabilization of misfolded proteins, co-chaperons for Hsp70
Hsp60	Mitochondria	Protein folding assembles multimeric complexes.
Hsp70	Cytosol/ Nucleus	Protein folding, membrane transport proteins.
Hsp90	Cytosol/ Nucleus	Regulatory interaction with signalling proteins, stabilization of misfolded proteins.
Hsp100	Cytosol/ Nucleus	Thermotolerance, protein refolding.

When *B. mori* is subjected to a temperature greater than normal, the minor heat shock proteins, namely BmHsp19.9, BmHsp21.4, BmHsp23.7, BmHsp27.4, and Hsp 1, are stimulated (Sakano *et al*., 2006). Furthermore, research has demonstrated the expression of a small Hsp in silkworms called BmHsp27.4, which has been found to express itself in response to extremely high temperatures (Wang *et al*., 2014b).

Reports showed that under extreme heat stress, the expression of proteins linked to the production of silk drastically decreased. On the other hand, compared to the normal condition, there was a significant increase in the expression of stress-related proteins (Li *et al*., 2012). It appears that silkworm breeds that are multi- and bivoltine react differentially to heat stress (Joy and Gopinathan, 1995).

1.9 Significance of Nutrition and diet in silkworm

Nutrition is the most crucial and necessary element for all living things. It is impossible for any living thing to thrive and grow in a healthy, disease-free state without nourishment or nourishing food. Silkworms are extremely sensitive to dietary factors, much as other creatures. When silkworms lack essential nutrients found in leaves, they become unhealthy and the proportion of successful rearing rates decreases. This is because poor-fed silkworms have a greater mortality rate. The chemical components of food that are necessary for an insect's proper growth and metabolism are known as nutritional needs (House, 1962). Generally speaking, insects need the same nutrients as huge mammals. For the majority of insects under study, the nutritional balance is crucial (House, 1965; Dadd, 1985).

Studies on the use of feed are often limited to the fourth and fifth instars of silkworm larvae since during these stages, 80% of the entire leaf mass is devoured. Feed conversion efficiency is regarded as a crucial physiological parameter for assessing the quality of silkworm breeds and directly or indirectly contributes to a significant portion of the cost-benefit ratio of maintaining silkworms (Junliang and Xiaofeng, 1992). The growth, development, food intake, utilisation, and conversion efficiency of different breeds of silkworms may differ. The environment, the quality, and the quantity of food significantly affect how efficiently silkworms convert food into silk thread (Mathur *et al*., 2002).

Silkworm *B. mori* only consumes mulberry leaves for growth and metabolism for the entirety of their existence. The nutritional content of mulberry leaves has a major impact on the development of the larvae and the production of cocoons. The total performance of silkworms throughout development is directly influenced by their nutritional physiology. For the normal growth of silkworm, it requires specific amino acids, sugars, proteins and vitamins. All of the nutrients required for *B. mori*'s co-evolution and natural selection are provided by mulberry leaves (Rajabi *et al*, 2007).

Mulberry leaves are biochemically composed of vitamins, proteins, minerals, lipids and carbohydrates. The nutritional content of mulberry leaves significantly affects the growth and metabolism of silkworms (Ravikumar. *et.al,* 1988). Additionally, it has been noted that the dietary value of food also has a significant impact on the release of digestive enzymes and assimilation and their survival rate (Bahar *et al*., 2011).

It is commonly recognised that the quantity and quality of leaves influence the growth rate, developmental stage, body weight, and survival rate of larvae. They also have an impact on the longevity, fecundity, movement and adaptations of the adult (Parra, 1991). The growth of healthy larvae and the production of superior cocoons depend on the choice of plant nourishment with exceptional nutritional value (Nijagal and Kumara, 2017). The impact of high-quality diet on the raising of B. mori has been well acknowledged, both domestically and internationally (Mendonça *et al*., 2010., Vadamalai, 2011; Sarkar, 2018). The host plants of Oak tasar (Sur *et al*., 2017), Tropical tasar (Ojha *et al*., 2009), Eri, (Debaraj *et al*., 2003; Sarmah *et al*., 2015) and Muga (Sarmah *et al*., 2015) have been used to evaluate the seasonal change of foliar nutrients in non-mulberry sericulture. The life cycle of silkworm is impacted by the digestion and absorption of the nutrients from mulberry leaves. To understand the interaction between the plant and the insect, a quantitative examination of the nutrition of the insect is necessary (Waldbauer, 1968). Since the amount of food taken and digested directly affects physiological processes and silk production, this in turn affects the quality of silk (Jyothi *et al*, 2014., Ruth *et al*., 2019)

1.10 Impact of fortification of nutrients on silkworm *B. mori*

All four species of silkworms—mulberry, tasar, muga, and eri obtain their necessary nutrition from the leaves they consume. To raise silkworms that would generate better-quality silk, researchers also attempted alternative food sources, but they were not financially viable. So, supplementation of nutrients, vitamins or minerals through the mulberry leaves can be used as an effective process to improve the quality and quantity of silk production. By adding vitamins and other elements to the leaves, their nutritional condition can be enhanced (Kanafi *et al*., 2017). Better growth and development of silkworm larvae, as well as a direct impact on the amount and quality of silk production, were demonstrated by feeding them with nutritionally enhanced leaves (Neto *et al*, 1995). All genetic features, including weight of the larvae and

cocoon, quantity of silk produced, pupation, and reproductive qualities, are directly impacted by the dietary requirements for nourishment (Khurram, 1998).

Ahmad (1993) discovered that combining various nutrients results in increased silk production and larval weight. Enriching mulberry leaves with certain proteins, amino acids, carbohydrates, and vitamins enhanced the amount and quality of silk produced (Etebari & Matindoost, 2005). Dietary protein supplements, such as those derived from black beans, soybeans, and mushrooms, have been linked to improve the growth traits and lower mortality (Mahmoud, 2013). When mulberry leaves were fortified with a certain quantity of the amino acids found in silk fibre, *B. mori* developed and matured more favourably (Radjabi, 2010). The economic features of *B. mori* were enhanced by the supplementation of thyroxine hormone (Latha *et al*., 2011) and their larval development was positively influenced by cyanocobalamin (Das and Medda, 1988), antibiotics (Ha *et al.*, 2017), thyroxine (Ahmad *et al*., 2009), methoprene (Miranda *et al*., 2002), vitamin B (Pal, 2003) and HCl supplementation (Gong *et al*, 2016).

Additionally, it has been shown that adding salt to the diet of silkworm *B. mori* boosts its biochemical content (Etebari and Fazilati, 2003). Larval weight increased three times when mulberry leaves combined with bovine milk were observed (Konala *et al*., 2013). Similar to this, silkworms fed on mulberry leaves treated with ammonia solution and farmyard manure formed heavier cocoons and considerably increased larval weight (Mahmood *et. al.,* 2002). Supplementing *B. mori* larvae with mestranol and norethindrone, juvenile hormone mimics, positively controlled their development (Mamatha *et al*., 2008., Cheng *et al*., 2014). Furthermore, feeding with 20-hydroxyecdysone had a beneficial impact (Park *et al*., 2003; Prasad and Upadhyay, 2012). The weight of the silk gland, the cocoon-shell ratio, the length and weight of the filament, and the amount of sericin and fibroin were all increased by the silver nanoparticles and spirulina feeding (Thangapandiyan and Dharanipriya, 2019). Furthermore, it has been suggested that the addition of vitamin B to the mulberry leaves may increase silkworms' resistance to environmental stress than the control larvae (Rahmathulla *et. al.*, 2002).

1.11 Effect of Vitamin C supplementation on silkworm

Vitamins are considered to be essential to the proper functioning of enzyme systems. They are necessary chemicals to modify cellular metabolism and physiological

function (Shamsuddin, 2009). For growth and development, the silkworm mostly needs ascorbic acid and vitamin B complex. It has long been believed that L-ascorbic acid, or vitamin C, is essential for *B. mori* growth and development. Mulberries do contain a significant quantity of ascorbic acid; however, they are unable to synthesise it (Bresci, 1951; Mosallanejad *et al*., 2002).

Vitamin C is considered as a vital nutrient for the development, repairing of tissue and functioning of several enzymes of silkworm. It serves a variety of vital purposes in the body of the silkworm. It is an effective antioxidant that protects against oxidative damage to proteins, membrane lipids, and DNA (Gomma *et al*., 1977). The lack of ascorbic acid in the food of silkworm larvae in their first and second instars delays their growth and development (Kanafi *et al*., 2007). Ascorbic acid is typically added to silkworm food (enrichment) in amounts that range from 1% to 2% of the artificial diet's dry weight, which is thought to be the ideal amount of this vitamin (Ito, 1961).

Research has shown that supplementing ascorbic acid to the diet of silkworm larvae significantly boosted their weight (Rajabi *et al*., 2006). Lower mortality and faster growth were observed in silkworms fed on mulberry leaves augmented with ascorbic acid and nitrogen (Javed and Gondal, 2002). Supplementation of Vitamin C also enhanced the commercial traits of silkworm cocoons (El- Karaksy and Idriss, 1990; Etebari & Matindoost, 2005). Protein content in the *B. mori* silk gland increased with vitamin C treatment (Brahma *et al*., 2018).

Since there is a growing market for finest silk, it is necessary to generate high-quality cocoons. From a sericulturist's perspective, this can only be done by selecting silkworms with high yields and improving the nutritional elements of the worms. The amount and quality of silk produced are directly impacted by the diet (Legay, 1958). A contemporary method for raising the cocoons' economic worth is to fortify the mulberry leaves with additional nutrients (Kumararaj *et al*., 1972). The current study employs a unique method to the nutritional management of silkworms, feeding the worms with vitamin C fortified mulberry leaves to ensure the generation of healthy silkworms and high-quality silk production even in the condition of high temperature.

Analysis of gut microbes in bivoltine silkworm *B. mori* may reveal the potential functionality in these microbes that may be required for the growth and development of the silkworm. One of the most important tools for researching the structure,

functions and evolution of diverse microbiota is genome analysis. We have focused on the application of 16S rRNA high throughput genome sequencing to study the gut microbiota of silkworms, as well as the latest developments in this area that aim to investigate the gut symbionts of insects. In addition to offering the first insights into every bacterial gene found in silkworm guts, these datasets aid in the generation of hypotheses that will be tested later on to determine the functional characteristics of the gut microbiota in a significant insect group. So, in this present study we propose to analyse the diversities of gut microflora of bivoltine silkworm *B. mori* on supplementation of ascorbic acid under thermal stress.

1.12 Objectives of the study

- To study the diversities of gut microflora of bivoltine silkworm *Bombyx mori* by
    1. Cultural characterization
    2. Chemical characterization
    3. Metagenomic analysis
- Comparative study of gut bacteria of bivoltine silkworm under thermal stress and ascorbic acid supplementation.
- To find out the impact of supplementation of ascorbic acid on the digestive enzymes of silkworm, *Bombyx mori* under thermal stress.
- Quality evaluation and morphometric parameters of cocoons.

1.13 Relevance of the Study

Silkworm *Bombyx mori* is an important insect that produces silk with significant economic value. Sericulture is one of the major agro based domesticated industries that offers financial stability to rural residents, particularly women and is encouraged as a secondary occupation. Due to the unfeasible climate in India for producing high-quality cocoons, a large number of farmers abandon their fields and the industry altogether. The high temperature due to global warming is adversely affecting the quality and quantity of cocoon. Since there is an increasing demand for premium silk, it is necessary to generate high-quality cocoons. From a sericulturist perspective, this can only be done by improving the nutritional aspects of silkworms. In order to help

farmers to rear silkworms in unfavourable weather conditions, researchers are now focusing on the nutritional supplement diet of the silkworm. One contemporary method for enhancing the economic value of cocoon is to fortify the mulberry leaves with additional nutrients (Kumararaj *et al*., 1972). The present research employs a unique method to the nutritional management of silkworms, by fortifying the mulberry leaves with vitamin C to ensure the generation of healthy silkworms and high-quality silk under thermal stress. All of these factors also affect an insect's physiology, which is managed by endosymbionts. The guts of silkworms offer exceptional conditions for the colonisation of microorganisms, particularly bacteria, which may offer several advantageous functions to their hosts. Study on gut microflora will advance our understanding of the function of bacteria in silkworm development and growth.

However, an attempt was made to determine the impact of vitamin C fortification in the growth of silkworm *Bombyx mori* under thermal stress. Relevant examinations were conducted to evaluate the growth characteristics and silk production capability of the gut bacteria of silkworms. The results of this study may be beneficial to the sericulture farmers of our country. Vitamin C has the potential to alleviate stress caused by environmental stress and can be utilised as a dietary supplement. Additionally, it is possible to produce silk on a large scale using inexpensive substrates and enhance both quality and quantity. Moreover, knowledge about the gut bacteria of silkworm helps in further exploration of specific bacterial species involved in digestion and absorption, understanding their responses to thermal stress and vitamin C supplementation, and optimizing food digestion in sericulture practices. This knowledge contributes to a broader understanding of insect-microbe interactions and their impact on digestive processes, paving the way for advancements in insect physiology and applied entomology. Given the significant impact of gut bacteria on silkworm health, researchers can explore strategies to modulate the silkworm microbiome for enhanced resilience. This could involve targeted interventions to promote the growth of beneficial bacteria or the use of probiotics to support gut health under stress conditions. A product that is ready for use could be formulated to support silk farmers. In collaboration with the central and state bodies for sericulture, field implementation may be made more widely accepted.

CHAPTER 2

RIVIEW OF LITERATURE

2. Review of literature

Sericulture, also known as silkworm farming, is the breeding of silkworms for the purpose of producing high quality and quantity of silk, which helps in the growth of nation's economy. Production of high-quality cocoons forms the basis of improved silk production. To accomplish this goal, cocoon yield should be increased by overcoming adverse climatic conditions and improving the nutritional diet. This entails vitamin fortification through their typical diet of mulberry leaves. The digestion of these nutrients and gut symbionts in the silkworm also play an important part in their development. Although other commercial silkworm species exist, *Bombyx mori* is the most frequently used and extensively studied silkworm. Several research studies on sericulture and cocoon production have been conducted, encompassing a wide range of aspects such as mulberry cultivation, silkworm rearing, the economics of cocoon production, biochemical and molecular modifications, microbiological characterization, and so on.

2.1. Silkworm as an experimental insect

Silkworm *Bombyx mori* is a lepidopteran model insect of significant agricultural and economic relevance. As stated by Xia *et al.,* (2004), the silkworm *B. mori* is "economically important" owing to its usage in the silk production process. As silkworms are extremely sensitive to climate change and pollution, they may be utilized as a key factor in environmental monitoring and as an ecological indicator (Sekimura., 2005, Hamamoto *et al.,* 2009). Silkworm is used as an important model organism in research because of its relatively large size, ease of handling, and simplicity of management and rearing (Xia *et al.,* 2014).

According to Meng *et al.,* (2017), the silkworm is an ideal model organism in biological research since it has its relatively short lifespan, high genetic makeup, precisely sequenced genome, and a substantial number of genes that are identical to silkworm, making it suitable for various life science studies. Panthee, *et al.,* (2017) specified the silkworm as an experimental model because it does not need ethical approval, does not require heavy and costly tools for rearing and considerable numbers of larvae can be raised in a single box, thereby lowering the expenditure of maintaining silkworms. Silkworm has been utilized as a model animal for the research

regarding microbial pathogenicity and the medicinal properties of antibiotics (Jha *et al.,* 2022).

2.2. Bacterial diversity in the silkworm gut

Insect gut is one of the most crucial organs in their body since digestion and absorption of nutrients takes place there which helps in the growth and metabolism of it. The principal functions of the gut are mediating the efficient digestion of the food and protecting the organism against harmful chemicals and pathogens. The gut bacteria of insects are vital for its growth, development, and ability to adapt to the environment. Changes in these organisms due to stress or environmental shifts can lead to unhealthy conditions and increase the insect's vulnerability to diseases. (Liang *et al.,* 2015). In contemporary times, there is a growing interest in understanding the diversity of these gut bacteria and their impact on the metabolism of silkworm to improve silk production. Exploring the microbial composition can offer valuable insights into the interaction between the insect and its gut bacteria, contributing positively to these processes. The growth, development, and environmental adaptation of the host insect are greatly influenced by the gut symbionts. Numerous bacteria that support metabolic activities are found in their gut; however, only few studies were reported about the diversity and its contribution to their host. The present study compares the gut bacterial diversity of *B. mori* on supplementation of vitamin C under thermal stress to study the influence of bacteria in the growth and development of silkworm larvae. Recognizing the pivotal role of gut bacteria in nutrient absorption and overall host health, our study highlights the potential effect of vitamin C under thermal stress, which is well-known for its antioxidant qualities and advantages in the stress response. The fact that the production of silk is heavily dependent on healthy silkworm larvae, this study aims to determine if changes in the gut microbiota brought on by heat stress and vitamin C have any discernible influence on the growth and development of the larvae. The results might help improve sericulture techniques while also offering new perspectives on the interactions between environmental stresses, host species, and the microbial populations that are vital to the health of insects.

According to the study of Kalpana. *et al.,* (1994), bacterial flora plays a significant role in the digestion of food material. Silkworm gut bacteria have been found to

produce some digestive enzymes such as amylase, caseinase, gelatinase, lipase, and urease. They investigated the changes that occur in the bacterial flora of the silkworm (*Bombyx mori*) during various phases of its life cycle. The results revealed a greater heterotrophic microbial community in the final instars, which correspond to the active consumption stage of larvae.

Feng *et al.,* (2011) identified fifty-six bacteria from the gut of silkworm larvae raised on tricuspid cudrania leaves. *Bacillus, Brevibacterium, Corynebacterium, Staphylococcus, Klebsiella,* and *Stenotrophomonas* were among the nine lipase-producing bacterial strains isolated and categorised into six genera. Their findings demonstrated that nutrition has a major influence on the gut bacterial population, particularly lipase-producing bacteria, and that differences in lipase-producing bacterial diversity may be associated with silkworm disease resistance.

Liang *et al.,* (2014) employed a bacterial culture method, metagenomic and denaturing gradient gel electrophoresis method to compare bacterial communities in the silkworm larval gut between the bioregenerative life support system rearing way and the traditional rearing way. The culture-dependent technique revealed a significantly lower count of silkworm gut bacteria in the BRW compared to the TRW. Analysis of clone libraries showed that the gut microbiota in the BRW was notably less diverse than that of the TRW. In the BRW, Acinetobacter and Bacteroides dominated, whereas the TRW was dominated by Bacillus and Arcobacter.

Sun *et al.,* (2016) studied the gut microbiota of the healthy silkworm *Bombyx mori* and alterations during *B. mori* cypovirus infection. The intestinal contents of control larvae and infected larvae included 147(135) and 113(103) genera, respectively. It was discovered that Enterococcus, Delftia, Pelomonas, Ralstonia, and Staphylococcus were the dominant bacteria obtained from the control. It was also noted that the diversity gut bacteria in the infected larvae decreased, whereas the abundance of both gram-positive bacteria Enterococcus and Staphylococcus were improved. Their results indicated that the observed variations in relative abundance were linked to the silkworm's immune response to BmCPV infection. Their research shed light on the association between gut microbiota and the development of BmCPV-infected silkworms.

Chen *et al.,* (2018) used next-generation sequencing (NGS)-based shotgun metagenomics to characterise the biodiversity and functional potential of the gut microbiota of the silkworm *Bombyx mori*. Two metagenomes were constructed, one for the conventional inbred strain Dazao (P50) and one for the enhanced hybrid strain Qiufeng Baiyu (QB), which is extensively utilised in commercial silk production and contains 45,505,084 and 69,127,002 raw reads, respectively. Their taxonomic study found that more than six hundred bacterial species were detected in inbred silkworms (P50), whereas 322 distinct species were identified in hybid silkworms (QB). Markedly, both strains were dominated by Enterobacter, Acinetobacter, and Enterococcus.

Chen *et al.,* (2020) administered various antibiotics to silkworm *Bombyx mori* to develop gut dysbiosis (microbiota imbalance) in order to evaluate the probable mechanisms by which the microbiota transmits pesticide resistance to silkworm. According to Li *et al.,* (2020) phoxim exposure to the silkworm larvae enhanced the evenness of the gut bacterial population and affected the structure of the gut microbiota. The study demonstrated a significant reduction in the abundances of specific taxa, such as Methylobacterium and Aurantimonadaceae, in the larval gut treated with phoxim compared to the water-treated group. Additionally, phoxim led to a decrease in the production of antimicrobial peptides and an enhancement in the pathogenicity of *Enterobacter cloacae* against silkworm larvae, indicating immune system suppression due to phoxim exposure. The research established that exposure to phoxim induced changes in the gut microbial community, and the resultant alterations in the composition and structure of intestinal microorganisms could impact the normal functioning of the silkworm's digestive tract. The findings underscored the significance of the gut microbiota in comprehending the underlying causes of midgut damage in *B. mori* resulting from exposure to pesticide.

Barretto *et al.,* (2021) explored the significance of intestinal bacteria in silkworms and their practical implications. They highlighted the crucial role of gut flora in ensuring the survival of insects by providing protection against predators and infectious agents through mechanisms such as colonial resistance, toxin production, and activation of the insect immune system to combat invading organisms. Given the essential functions of gut microflora in aiding the insect host, these bacteria present

opportunities for applications in various industries, including medical sciences and agriculture.

Dee & Bautista (2022) have examined the features affecting the growth and development of silkworm larvae and cocoon characteristics which in turn affect the yield and quality of silk. It was highlighted that the gut microbiota has been associated with absorption and utilisation of nutrients, as well as immunity to illnesses, and has been shown to influence silkworm growth and development. Metagenome sequencing was employed to profile the bacterial population in four native silkworm strains cultivated in the Philippines region. The results showed the prevalence of bacteria from the genera Pseudomonas, Sphingomonas, Delftia, Methylobacterium, and Acinetobacter across all four silkworm strains. Furthermore, the findings demonstrated an augmentation in bacterial diversity and evenness as the larvae progressed in their developmental stages, suggesting a correlation with larval growth and variations in the quantity and maturity of mulberry leaves consumed by the larvae.

Utilizing blast analysis, Sharmila *et al*. (2023) identified the primary bacterial species within the digestive tracts of both healthy and unhealthy groups of Pure Mysore and FC1xFC2. The investigation also unveiled the existence of *Proteus sps*, *Flavobacterium sps*, *Bacillus* and *Pseudomonas* through genome sequencing, while Klebsiella and Enterobacter gut bacteria were discerned through biochemical characterization. The studies demonstrated the presence of the mentioned bacterial species in the gut of silkworm breeds.

Yeruva *et al.,* (2020) compared the diversity of midgut bacterial communities in silkworms of variable voltinism (Pure Mysore, PM: multivoltine; CSR2: bivoltine and PM × CSR2: crossbreed) through metagenomics. In PM, Enterococcus was prevalent, followed by Bacillus, while CSR2 showed a predominance of Lactobacillus followed by Enterococcus. Interestingly, the midgut of the crossbreed exhibited a diverse bacterial community including Bacillus, Lactobacillus, Enterococcus, and uncultured bacteria. The metagenomic profiles highlighted the variability in gut bacterial populations among different silkworm varieties, influencing physiological activities accordingly.

2.3. Digestive enzymes synthesised by bacteria

Insect gut has a wide range of enzymes, including digestive enzymes such as amylases, cellulases, ligninases, and pectinases, lipases and proteases. These insect gut symbionts also provide critical nutrients to the insect host, such as vitamins, amino acids, and sterols. and it serves as a source of various enzymes.

Many studies reported lipases and proteases from a variety of insect gut symbionts, including Bacillus, Staphylococcus, Pseudomonas, Fusarium solani, *Candida fermentati,* and *Yarrowinia lipolytica* from silkworms and beetles (Park *et al.,* 2007; Feng *et al.,* 2011).

Prem *et al.,* (2010) reported the existence of bacterial species capable of degrading cellulose, xylan, pectin, and starch consumed by the silkworm *B. mori. Proteus vulgaris, Klebsiella pneumoniae*, and *Citrobacter freundii* were isolated and are capable of breaking down cellulose and xylan. Pectin was utilised by Erwinia sp. and *Pseudomonas fluorescens*. *K. pneumoniae* could break down cellulose, xylan, and starch. *Serratia liquefaciens* was able to degrade pectin, xylan, and cellulose. It is also revealed that, with every instar, the quantity of cellulolytic bacteria increased.

Sun and Scharf (2010) reported a large number of gut symbionts associated with insects that help in the digestion of lignocellulosic material into glucose molecules. As stated by Khyade and Marathe (2012), the midgut of *B. mori'* contain cellulolytic bacteria that aid in digestion, absorption and growth. In the study by Fischer *et al.,* (2013), a range of bacteria with cellulolytic activity were isolated from the gut of lepidopterans, including Pseudomonas spp., Enterobacter sp., *Klebsiella pneumoniae,* and *Proteus mirabilis*. Poulsen *et al.,* (2014) have reported the isolation of new cellulose enzymes that break down plant fibres and proteins.

Liang *et al.,* (2015) used Illumina Miseq sequencing and a culture-dependent approach to compare the variations in gut bacterial diversity of the silkworm larvae and cellulase-producing and amylase-producing bacteria between the BLSS rearing method (BRW) and the traditional rearing method (TRW). The research indicated that isolates from silkworm gut bacteria in the BRW may produce enzymes capable of degrading cellulose and starch. However, it was noted that the number of isolates releasing cellulase and amylase is the same. Alternaria sp., Preussia sp., and

Coprinellus radians were the isolates that could synthesise both enzymes in the TRW. Meanwhile, they reported that Enterococcus, Erwinia, and Pantoea could generate cellulase and amylase in the BRW.

Gandotra et al. (2018) utilized culture-dependent techniques, generic identification via 16S ribosomal RNA probes, and qualitative screening for enzyme activities to identify *Bacillus spp., Serratia marcescens, Stenotrophomonas maltophilia, Pseudomonas stutzeri, Acinetobacter sp*., and *Alcaligens sp*. residing in the gut of the muga silkworm. Their findings revealed that Bacillus (54%) was the predominant bacterial genus in the culturable bacterial community of *A. assamensis*, followed by Serratia, Pseudomonas, and Alcaligens. Through qualitative enzyme activity screening, approximately twentyfive gut bacterial isolates exhibited significantly increased amylase, cellulose, and lipase activities, indicating their potential role in aiding the digestion and feeding of their host insect.

2.4. Impact of Thermal Stress on Sericulture

The success of sericulture is influenced by their environmental factors, especially temperature, when increases beyond the optimum it may adversely affect the growth of silkworm larvae. The abiotic factors such as temperature, humidity, light and nutrition have an impact on the phenotype of silkworm larvae (Pillai *et al.,* 1980; Tazima, 1984). Several studies reported that fourth and fifth instars of silkworm larvae were highly sensitive to high temperature t (Ueda and Lizuka, 1962; Shirota, 1992; Tazima and Ohuma, 1995). Ueda *et al.,* (1975) and Benchamin and Jolly (1986) reported that temperature is a crucial abiotic factor that influences the development and cocoon production in silkworm. Fine quality of silk is produced when the silkworm is reared at an optimum temperature of 22 -27° C (Krishnaswamy *et al.,* 1973). Bivoltine silkworms are better tolerable than univoltine and multivoltine races to varied climatic conditions (Rao, 1998).

Ramachandra *et al.,* (2001) reported that the rate of spinning of silkworm *Bombyx mori* larvae was slow at 22°C and fast at 38°C. Good quality of cocoons were produced from slow spinning larvae which were reared at 22°C than the fast-spinning cocoons reared under 38°C. They concluded that high temperature affects the quality and quantity of cocoon production and recommended 22° C is the optimum temperature for silkworm rearing.

The effects of climate change on the gut-associated symbionts of two stink bugs, *Acrosternum hilare* and *Murgantia histrionica*, were investigated by Prado *et al.,* (2010). Using diagnostic PCR primer sets, they monitored on the existence of gut-associated symbionts and demographic data of two species reared at two constant temperatures, 25 and 30°C. As per their results, both bugs experienced a loss of their gut microbes across two generations at 30°C. Additionally, at 30°C, both *A. hilare* and *M. histrionica* displayed reduced insect survivability and reproduction rates compared to those observed at 25°C. The high temperature also seemed to be lowering the general fitness of insects, according to other demographic characteristics. All of their results indicated that at 30°C, symbiont loss was associated with and maybe caused by a decline in host fitness.

Rahmathulla, (2012) emphasised that as silkworms are cold-blooded insects, temperature directly affects a number of physiological processes, including the development of the worms. According to him, early instar larvae can withstand high temperatures, which enhances their chances of survival and the characteristics of their cocoons. The growth of silkworms is directly correlated with temperature; large temperature fluctuations are detrimental to silkworm development. When the temperature rises, a number of physiological processes increase, and when the temperature drops, fewer physiological processes occur. Rearing silkworms at a higher temperature speeds up larval growth and shortens the larval period, especially in the later instars. On the other side, growth is slower and the larval stage is lengthened at cold temperatures.

Potential bivoltine silkworm strains with a particular resistance to high temperatures were found by Sugnana *et al.,* (2011). It has been asserted that in majority of India's sericulture sites, summer temperature rises have a negative impact on the development of temperate bivoltine silkworms and resulted in crop losses. Therefore, screening silkworms for thermotolerance is a crucial step in the development of breeds and hybrids that are thermotolerant. On their investigation, bivoltine strains of silkworm larvae on their third day of the fifth stage were exposed to a high temperature of 36 ± 1 °C and a relative humidity of 50 ± 5% for six hours each day (10:00–16:00) till their spinning. All genetic features of bivoltine silkworm strains in the treatment groups showed highly significant differences.

Hussain, (2011) studied the effects of temperature and humidity variations on pupation, hatchability, and larval mortality of eleven inbred silkworm lines: M-101, M–103, M–104, M–107, Pak-1, Pak–2, Pak–3, Pak–4, PFI–I, PFI–II, and S-1. Three different temperature conditions (25, 30, and 35 ± 1°C) were applied to larvae in their fourth and fifth instars. Temperature and relative humidity had a significant impact on the hatchability, pupation, and mortality of inbred silkworm lines of fourth and fifth instar larvae. According to their findings, during the fourth and fifth instars, the mean performance of inbred silkworm lines varied considerably from one another under diverse temperature and humidity exposures. The performance of silkworm lines was constant at 25°C and 75% relative humidity, but changes in either temperature or humidity for three hours had a substantial impact on all three evaluates (hatchability, pupation, and larval mortality).

The impact of the environment (a range of temperatures and relative humidities) on the morphology, mechanical characteristics, and physical characteristics of the cocoon and spinning pattern of the silkworm *Bombyx mori* were investigated by Offord *et al.,* (2016). The stiffness and strength of the cocoon are specifically influenced by temperature, which attribute to changed spinning behaviour and sericin curing time. Perhaps as a result of tanning compounds, relative humidity has an impact on cocoon colouration. Furthermore, a cocoon's water content affects stiffness and sericin dispersion without altering toughness. Their findings showed that environmental quality criteria must be regarded while studying and using silk cocoons, silk filaments, or silk-derived biopolymers.

Sarkhel *et al.,* (2017) deduced from her review that temperature affects the mulberry silkworm's yields and survival rate. Temperature and the different characteristics of silkworm larvae are negatively correlated. According to their studies, silkworm larvae grown and developed at optimal temperatures of 22–26°C and 75–85% relative humidity will grow and develop more, which will increase sericulture productivity. Larval weight often decreases with increasing temperature. Elevated temperatures hindered productivity and caused mulberry leaves fed to silkworms to wither, which decreased the amount of food they received. However, high temperatures fasten the rate of development, which results in low-quality cocoons.

Sun *et al.,* (2017) examined the alterations in the intestinal flora at 48, 96, and 144 hours after 8 hours of transient high temperature treatment (THTT) at 37°C. Principal analysis showed a negative association between the abundances of *Enterococcus* and *Staphylococcus* with other prevalent genera. After THTT, there was an increase in spatzle-1 transcript levels and a decrease in dicer-2 levels, suggesting activation of the Toll pathway and suppression of the RNAi pathway. The spatzle-1 gene expression level displayed a negative correlation with the community richness of *Enterococcus*, while a positive correlation was observed between dicer-2 and the abundance of Variovorax post-THTT. Their research yielded novel insights into the connections between intestinal flora, host gene expression, and THTT.

Sun *et al.,* (2022) used high throughput sequencing and biochemical tests to identify silkworm gut bacteria treated with high temperature for 72 hours in order to study the impact of intestinal microbes on silkworms in response to a high-temperature environment. The findings demonstrated that gut bacteria of silkworm larvae were impacted by high temperatures and that there are gender variations, with females being more susceptible. Gut bacteria are connected to alterations in the metabolism and transport capacity of silkworm tissue in the gut at high temperatures. Elevated temperatures have the potential to impact silkworm gut microbiota by controlling the function of relevant digestive enzymes and intestinal substance transport. This, in turn, might impact the silkworm's nutritional absorption and digestion, eventually impacting its development and growth.

2.5. Heat shock proteins (Hsps)

The multivoltine mulberry silkworm, *Bombyx mori* of the Nistari race, was subjected to an experimental examination by Sinha and Sanyal (2013) to determine its heat sensitivity. In this investigation, the efficacy of silkworm eggs, larvae, pupae, and adults at 17°, 33°, and 43° Celsius was assessed. The weight of the cocoon and its shell increased significantly following heat stress in nistari as compared to the control. This is hypothesized to be attributed to the expression of heat shock proteins (Hsp) during the larval period. These findings suggest that a breeding strategy based on heat shock proteins should be employed to enhance the resilience of breeding stock intended for industrial purposes.

The survival and heat shock protein 90 expression patterns in *Bombyx mori* have been reported by Keshan *et al.,* (2014). During its larval development, the fat body, wing disc, and dorsal abdominal epidermis, as well as the ovary during its pupal stage, all showed widespread expression of Hsp90 mRNA. Its expression was markedly up-regulated in response to mild and mild to severe heat stress (39°C and 42°C), with the exception of pupal ovaries. However, it was shown that the overexpression of Hsp90 was connected with an animal's ability to withstand the particular heat shock temperature. It was observed that the spinning larvae were more resilient to heat stress than the pupae.

According to Chandrakanth *et al.,* (2015), there was a correlation between the three breeds' ratio and pupation during high temperatures and the expression levels of Hsp's during heat shock. The degree to which Hsp's are expressed is thought to be crucial for improving silkworm larvae's ability to survive at higher temperatures. Their studies indicate that the bivoltine breeds Hsp gene expression was higher than that of CSR2. Among the bivoltine breeds, it is the most tolerant to high temperatures in terms of pupation percentage.

Punyavathi, & Manjunatha (2020) reported that distinct male and female larval instars were subjected to differing levels of temperature stress on an individual basis. In particular, their sex-stage-specific protein expression was studied to decipher this cryptic characteristic. An immunoblot analysis revealed distinct variations in the expression of heat shock proteins 90 and 70 between male and female silkworm larvae across all instars. Notably, this study identified, for the first time, a significant correlation in the expression of the BmHsp90 and BmHsp70 genes in response to heat stress. This correlation, beyond highlighting sex-related differences, showcased a clear and coordinated impact on the survival and biosynthetic capabilities of *Bombyx mori* larvae throughout various developmental stages. Fang *et al*. (2021) extensively mapped the presence of heat shock proteins in 70 families across the entire silkworm genome. Specifically, the up-regulation of Hsp70-1, Hsp70-2, and Hsp70-3 was observed in response to both cold (2°C) and hot (37 and 42°C) stimuli, revealing the versatility of these proteins in responding to temperature-related stressors.

2.6. Importance of Nutrition in Silkworm

The essential elements necessary for silkworm growth are derived from the leaves that they consume as a diet. The nutritional quality of the leaves can be increased through vitamin enrichment (Kanafi *et al.,* 2007). The consumption of nutritionally enhanced leaves resulted in improved silkworm larval development and proliferation, as well as a direct effect on the quality and quantity of cocoon yield (Ito and Nirminura, 1966). Nutrition is an important factor in effective silkworm rearing, and hence a greater emphasis on silkworm nutrition (Borah and Boro, 2020).

Supplementation of mulberry leaves with vitamin B boosted silkworm resistance to unfavourable environmental conditions and it might increase nucleic acid and protein synthesis in the silk gland (Das and Medda, 1988). According to Chamudeswari and Radhakrishnaiah (1994), zinc plays a crucial role in larval metabolism by improved enzyme activities, hormone mediation, replication, transcription, and neural activity in silkworm. It has also been observed that a combined impact of 0.2% N + 0.1% P + 0.3% K and 0.1% Ca has a stunning effect on silkworm development and silk quality (Khurram, 1998). Javed and Gondal, (2002) has been found that when silkworm larvae were fed with 0.2% N treated mulberry leaves, the cocoon weight increased significantly. Suprakash and Pal (2002) discovered that vitamin B complex boosted the development and growth of silkworm as well as the economic aspects of the cocoon. The addition of copper sulphate, nickel chloride, and potassium iodide to the mulberry leaves boosted the silkworm's economic characteristics (Rezuanul *et al.,* 2004). Similar result has also been reported by Cristina and Marghitas, (2011) that showed an enhancement in the body weight and yield of silk production when the leaf was supplemented with vitamin B derivatives.

In the latter stages of multivoltine and bivoltine silkworms, *Bombyx mori*, Vyjayanthi, and Subramanyam, (2002) examined the alterations in the activity of a few digestive enzymes following fenvalerate-20 EC treatment. Enzymes including amylase, sucrase, and protease showed decreased activity after insecticide treatment, but trehalase activity increased when the midgut was utilised as the source of enzyme. The alterations observed in the activity of these enzymes suggested that fenvalerate may have detrimental effects on silkworm metabolism by interfering with the digestive enzymes' ability to work properly.

In the study conducted by Konala *et al*. (2013), it was revealed that feeding mulberry leaves soaked in bovine milk to larvae resulted in an 82.5% greater weight gain by the end of the fifth instar compared to larvae fed with fresh mulberry leaves without milk. During the initial 7 days of the fifth instar, the larvae consuming milk-treated leaves exhibited a remarkable 310% increase in their body weight, while those fed fresh leaves gained 153%. Additionally, the introduction of milk led to an 8% increase in cocoon weight compared to the control group. These findings suggest that supplementing mulberry leaves with nutrients, particularly through milk, could be a beneficial approach for enhancing the growth rate and silk production in B. mori larvae.

Based on Borgohain (2015) artificial feeds are helpful in raising protein and fat content, as well as improving the overall development and growth of silkworm larvae. Treatment of silkworm larvae with nutritional supplement improves the quality of silk filament, which may be utilised to increase production of silk. The findings of Kamaraj *et al.,* (2017), reported that feeding soya protein enriched leaves to silkworm larvae may improve development and boost the quality and quantity of cocoon production.

According to Saad (2019), feeding black seed and basil leaf extracts to experimentally infected silkworm larvae of *B. mori* with *Bacillus thuringiensis* resulted in a considerable rise in larval weight and a decrease in larval mortality. Furthermore, pupal weight was raised by black seed and basil leaves and the use of basil leaf extract resulted in a substantial increase in both cocoon weight and cocoon shell weight. When compared to the infected control, the silk ratios of the resulting cocoons increased at all tested doses of black seed extract.

Islam *et al.,* (2020) pointed out that, silkworm nutrition plays a significant effect in the growth and development of silkworm larvae, which is then reflected in the cocoon and reeling characteristics of the silkworm, *Bombyx mori* L. After the third moult, the silkworm double hybrid (CSR6 CSR26) (CSR2 CSR27) was fed on mulberry leaves reinforced with aqueous egg albumen solution. They found that egg albumen increased average filament length and raw silk percentage compared to the control.

When compared to the respective control, Riaz *et al.,* (2020) observed an improvement in the economic performance of double strains (FC1 × FC2) of silkworm

larvae based on larval, cocoon, pupa shell weights, and average shell ratio percentage relationship of nutrient intake in organic and inorganic diet.

2.7. Vitamin C as food supplementation

Vitamins are organic active chemicals that are required by cells to regulate their physiological function and metabolism (Shamsuddin, 2009). L-ascorbic acid (vitamin C) has historically been recognised as critical for the growth and development of *Bombyx mori* and serves several vital roles in the silkworm body. It is a potent antioxidant that protects DNA, membrane lipids, and proteins from oxidative damage (Gomma *et al*., 1977). In reality, ascorbic acid is abundant in mulberry leaves (Bresci,, 1951). However, the absence of ascorbic acid in the diet of silkworm larvae delays silkworm growth and development (Kanafi *et al.,* 2007).

Ito (1961) observed that ascorbic acid has a stimulatory impact on silkworm active eating during the first instar stage. Sengupta *et al.,* (1972) proposed that feeding silkworms with 1% ascorbic acid in mulberry leaves increased cocoon production. According to El-Karaksy & Idriss (1990), the impact of ascorbic acid as a food supplement drastically improved cocoon yield and output. Babu *et al.,* (1992) reported that leaf intake was higher in ascorbic acid supplemented leaves than in control leaves; essentially, vitamin C content of tissues and bodily fluids is greatly reliant on the quantity taken with food. Sarker *et al.,* (1995) found that supplementing mulberry leaves provided to silkworms with 1% ascorbic acid and 0.5% vitamin B complex increased cocoon production and silk filament quality. In the opinion of Etebari and Fazilati (2003), ascorbic acid increased the lifespan of silkworm larvae. According to Rajabi *et al*., (2007), ascorbic acid enhanced the weight of silkworm larvae significantly.

Zannoon *et al.,* (2008) enriched the leaves with nutritional additives such as vitamins C (1%) and B (0.2%), and three types of honey (from clover, cotton and citrus, 50%) and observed a significant enhancement in all tested groups compared with the control, especially for vitamin C and clover and citrus honey. The researchers suggested that fortification with either vitamins or bee-honey is extremely advantageous for the development of silkworm larvae and cocoon production.

Tantray & Trivedy (2011) supplemented mulberry leaves with seven different doses of vitamin C and were fed to 5th instar larvae of *Bombyx mori* at seven varied application schedules. The inclusion of 0.5% Vitamin C was observed to enhance larval weight, pupation rate, and cocoon production compared to the control when administered daily from 0 hours to the initiation of spinning.

Balasundaram *et al.,* (2013) observed that 0.2% Vitamin C treated group improved feed efficiency as well as the development of fifth instar silkworm larvae compared to control and other Vitamin C treated groups (0.1%, 0.4%, and 0.8%). According to this study, Vitamin C has some growth stimulant effect and may be employed to boost feed efficacy in commercial silkworm rearing with regard to sericulture.

Brahma *et al.,* (2018) observed an increase in larval body weight, silk gland weight, and silk gland protein content in vitamin C supplemented larvae compared to vitamin E supplementation.

2.8. Nutrition affecting gut bacteria of insects

Dong *et al*. (2018) found notable differences in gut microbial diversity and dominant bacteria between silkworms on an artificial diet supplemented with specific nutrients and those on a control diet. It also affected the development and metabolism of silkworm larvae. The gut microbial diversity was lower in the larvae reared on artificial diet. 16 S rRNA high throughput sequencing was used for the sequencing of gut bacteria. The report showed that, Cyanobacteria, Firmicutes, Proteobacteria, Bacteroidetes and Actinobacteria are the dominant bacteria in the silkworm larval gut of all the strains.

Chen *et al.,* (2023) evaluated the effect of endogenous Cd- polluted mulberry leaves on the gut bacteria of the silkworm and the outcomes revealed a dramatic variation in the gut bacteria of silkworm. A significant alteration in the richness of dominant phyla of silkworm gut was noted. Following exposure to Cadmium (Cd), a noteworthy rise in the prevalence of *Enterococcus, Brachybacterium,* and *Brevibacterium* groups associated with disease resistance at the genus level was observed. Simultaneously, there was an increase in the abundance of *Sphingomonas, Glutamicibacter,* and *Thermus*, which are linked to metal detoxification. Additionally, there was a

significant reduction in the abundance of Serratia and Enterobacter, pathogenic bacteria.

In conclusion, the review of literature has provided a comprehensive examination of the essential roles of gut bacteria, thermal stress, nutrient supplementation, digestive enzymes, and heat shock proteins in the development of silkworm, *Bombyx mori*. The negative impact of temperature on silkworm growth has been highlighted, emphasizing the significance of understanding and addressing this factor in the sericulture industry. Moreover, the importance of nutrient supplementation, digestive enzymes produced by bacteria, and the role of heat shock proteins have been underscored in relation to cocoon production. This study primarily focuses on the diversity of gut bacteria, investigating its impact on the growth and development of silkworm on supplementation of vitamin C under thermal stress.

CHAPTER 3

MATERIALS AND METHODS

3. Materials and Methods

3.1. EXPERIMENTAL INSECT

Scientific Classification

Kingdom	:	Animalia
Phylum	:	Arthropoda
Class	:	Insecta
Order	:	Lepidoptera
Family	:	Bombycidae
Genus	:	Bombyx
Species	:	*B. mori*
Binomial Name:		*Bombyx mori* L.

Fig 3.1. Silkworm *Bombyx mori* L.

3.2. PROCUREMENT OF SILKWORM

The silkworm *Bombyx mori* strain CSR26 was used as the experimental animal for this study. The DFLs (Disease Free Laying eggs) were collected from P2 Basic seed farm, Central Silk board, Pallatheri, Kerala, India. The silkworm larvae were reared and maintained in the laboratory condition. Since silkworms are highly sensitive, the rearing room needs to be properly ventilated in order to maintain the optimum temperature and humidity. Moisture, exposure to direct sunlight, and strong winds should also be avoided. The equipment and rearing room were properly cleaned and disinfected using a 2% formaldehyde solution. Chopped tender mulberry leaves were sprinkled over the egg cards and the hatched larvae were brushed out from the egg cards into rearing trays. The silkworm larvae were fed with fresh mulberry leaves *ad libitum* and maintained at optimum temperature (25 ± 2° C) and humidity (80 ± 5%) under a light and dark schedule of 12-hour duration (Krishnaswamy, 1978). Temperature and humidity were checked regularly using Thermometer and Hygrometer.

3.2.1. COLLECTION AND FEEDING OF MULBERRY LEAVES

Systematic Position of Mulberry leaves (V-1 variety)

Phylum	:	Dicotyledoneae
Sub Class	:	Monoclamidae
Order	:	Rosales
Family	:	Moraceae
Genus	:	Morus
Species	:	alba
Binomial Name:		*Morus alba* L.

Fig. 3.2. *Morus alba* L.

Mulberry silkworms exclusively feeds on mulberry leaves *Morus alba* L. The V-1 variety mulberry leaves were collected from the department mulberry garden to feed the silkworm throughout the period. The leaves were washed thoroughly to remove dirt and dust particles and were air dried before feeding. Only good quality leaves without any damages were fed to the larvae. They were fed 3 to 4 times a day. During the moulting period no feeding was given to them. Finely chopped tender mulberry leaves were used for feeding to the newly hatched larvae. Mature leaves collected from the bottom of the plant were used for feeding to the late instars. The feeding stage of silkworms are divided into seven phases. Initial feeding stage, the sparse eating stage, the moderate eating stage, the active eating stage, the premoulting stage, final feeding stage and the moulting stage. During the active feeding stage to final feeding stage, the larvae feeds voraciously and after that they stop feeding. The bed was cleaned regularly to remove leftover leaves, worms and faecal matter to maintain disease free healthy larvae.

3.2.2. Feeding Schedule

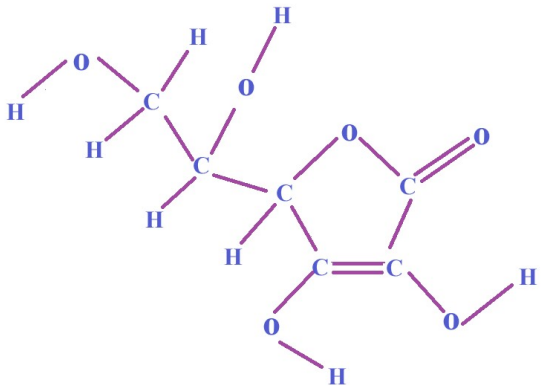
Table 3.1. Feeding Schedule of silkworm larvae

Instars	Time Interval				
1st Instar	9am	2pm	7pm	-	-
2nd Instar	9am	2pm	7pm	-	-
3rd Instar	8am	11am	2pm	5pm	8pm
4th Instar	8am	11am	2pm	5pm	8pm
5th Instar	8am	11am	2pm	5pm	8pm

3.3. EXPERIMENTAL CHEMICAL

Fig. 3.3. L- Ascorbic Acid (Vitamin C)

Properties of ascorbic acid



Chemical Formula : $C_6H_8O_6$

Molecular weight : 176.12 g/mol

Density : 1.694 g/cm^3

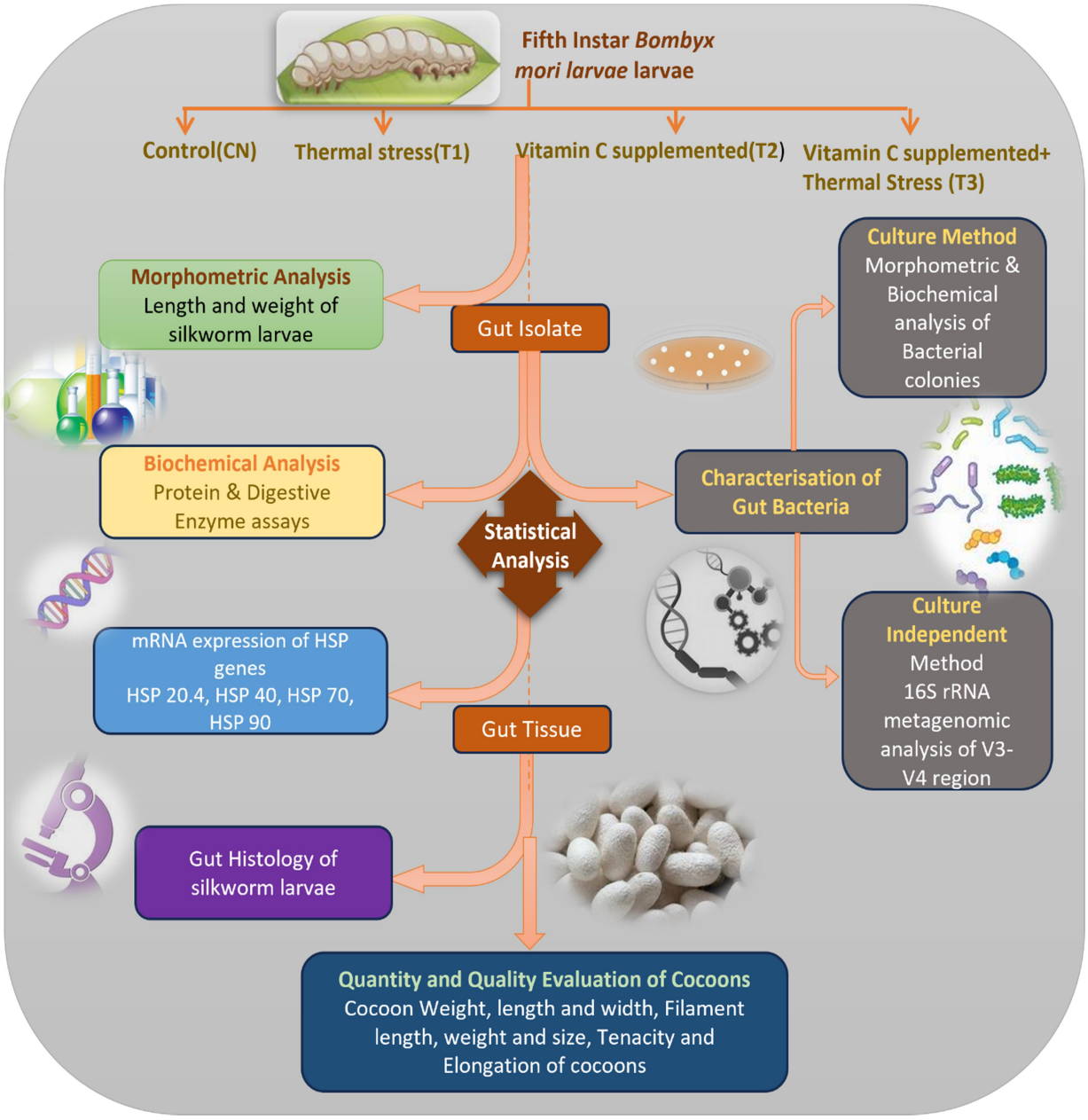
Boiling Point : 553°C

Melting Point : 190°C

Solubility : Water soluble

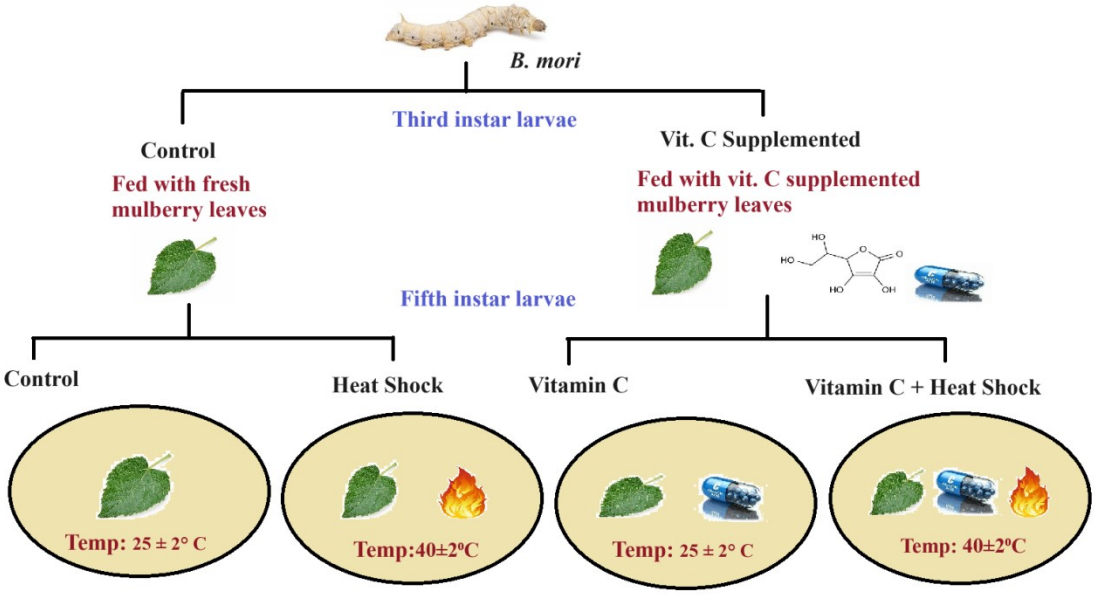
In the fortification of mulberry leaves, 0.2% concentration of vitamin C was used. This percentage was established as the standard during the previous experiments conducted in our laboratory (Aneesha, 2022).

Figure 3.4. Overview of methodology adopted



3.4. EXPERIMENTAL DESIGN

Figure 3.5. Experimental Design adopted for the study



Initially the newly exuviated third instar larvae were divided into two groups. One group was maintained as control fed with fresh mulberry leaves *ad libitum* and other group was fed with ascorbic acid supplemented mulberry leaves providing optimum temperature and humidity. The leaves were soaked in 0.2% ascorbic acid solution (dissolving 2grams of ascorbic acid in 2 litres of distilled water) for 1 hour and air dried the leaves and then fed to the larvae. After the fourth moult the fifth instar larvae were divided into four groups. One group served as the control and was exclusively provided with fresh mulberry leaves for feeding. Second group was exposed to heat shock (40±2ºC) for 1 hour per day and fed fresh mulberry leaves. Third and fourth group were fed with fresh mulberry leaves soaked in 0.2% ascorbic acid solution. Fourth group was exposed to temperature along with 0.2% ascorbic acid supplementation.

Experimental design

- Group 1 – CN – Control
- Group 2 – T1 – Heat shock exposed
- Group 3 – T2 – Ascorbic Acid Supplemented
- Group 4 – T3 – Heat shock with Ascorbic acid supplemented

3.5. CULTURE DEPENDENT MICROBIAL ANALYSIS

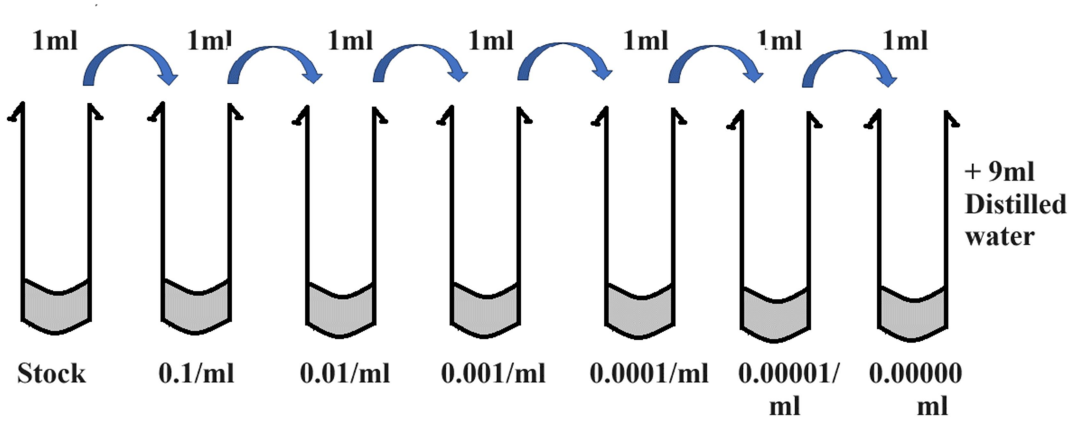
3.5.1. Extraction of Gut tissue.

Surface of the larvae were sterilised with 75% ethanol and washed with sterile distilled water. In the laminar airflow chamber, larvae (control, Hs, Vit C, and Hs+ Vit. C) were sacrificed in sterile ice-cold insect ringer solution (0.9) % and the gut was collected. The whole gut was taken to isolate bacteria. For further investigation, the gut tissues were homogenised using sterile, prechilled PBS in a sterile, prechilled mortar and pestle. The PBS solution (250mg/ml) was added based on the weight of the tissues. Homogenised and incubated at 37°C for 30 minutes. The supernatant was collected and the precipitate was discarded (Caporaso *et al*., 2010).

3.5.2. Screening of bacterial colonies.

The gut homogenates were diluted serially from 10 to 10^{-6} dilutions. For the spread plate technique on nutrient agar, 100µl of the diluted sample from 6 dilutions was obtained. The plates were incubated at 37℃ for 24 to 48 hours. The 10^{-5} dilution plates were chosen for further identification as they produced 30-300 bacterial colonies. The colony counter was used to count the solitary colonies. The morphology of colonies as well as their cultural characteristics were investigated. The pure culture colonies were subcultured on nutrient agar medium for further analysis (Broderick and Lemaiture, 2012).

Figure 3.6. Serial dilution of extracted bacterial sample



3.5.3. Identification of bacteria

3.5.3. i. Morphology and colony characteristics

Bacterial colonies were picked from nutrient agar plates based on morphological variations. The selected colonies were analysed for morphological characteristics such as colony colour, form, elevation, and margin, and then gram staining was performed on the bacterial cells to study their characteristics using a light binocular microscope (Leica ICC50 E).

3.5.3. ii. Gram staining

A small drop of bacterial culture was placed on a clean glass slide and spread it as a thin film and allowed the smear to air dry. The smear was flooded with crystal violet for 1 minute. After washing the excess stain, the smear was flooded with Gram's iodine for 1 minute. Then decolourised the smear with alcohol and washed it with distilled water. Safranin stain was added to the smear and kept for 1 minute. Excess stain was removed and air dried. The bacterial cell was identified using light binocular microscope (Leica ICC50 E). Purple coloured cells were Gram positive bacteria and pinkish red coloured cells represent gram negative bacteria.

3.5.4. Biochemical analysis

Selected colonies from control and treated groups (CN, HS, VC and HS+VC) were identified after performing the basic biochemical identification tests and compared the results with Bergey's manual of systemic bacteriology (1994) upto the genus level to determine the identity of the bacterial isolates. The biochemical tests performed were mentioned in the table below.

Table 3.2. Biochemical Characterisation of Gut Bacteria

Biochemical Test	Positive Result	Negative Result
Indole test	The red layer at the top of the tube	No colour change at the top of the tube
Methyl red test	Red colour formation	No change in colour
Voges Proskauer test	Red brown colour development	No chnge in colour
Citrate test	Colour change from green to Prussian blue	Green colour
Urease test	Pink colour	No change in colour
Catalase test	Presence of gas bubble	Absence of gas bubble
Oxidase test	A deep blue or purple colour in inoculated area of paper within 10-30 seconds.	No colour formation
H_2S production test	Black colouration along the line of stab	No black colour
Nitrate reduction test	Red colour formation	No change in colour
Gelatin liquefaction test	Liquification of the gelatin	No change
Starch Hydrolysis test	A clear zone around the test bacterial isolate	No change
Carbohydrate fermentation test	A red colour formation with or without gas production.	No colour change and gas production.

3.6. METAGENOMIC ANALYSIS (Culture independent method)

3.6.1. Isolation of bacterial DNA

Ten silkworm larvae were randomly chosen from each group, sterilized with 70% ethanol, and washed three times with distilled water (ddH2O). The complete gut dissected from the fifth instar larvae was utilized for bacterial genome isolation. Tissue samples from each group were combined and homogenized in liquid nitrogen using a sterile mortar and pestle, with triplicates taken for each sample. The bacterial genome was extracted using the JetFlex genomic DNA Purification Kit (Invitrogen) according to the manufacturer's protocol, and the entire procedure was carried out in a sterile environment within the laminar flow(Yuan *et al*., 2006).

The extracted DNA was subsequently employed for Illumina High-Throughput sequencing, and both the quality and quantity of the extracted DNA were assessed using Nanodrop.

Equipments Required

Mortar and pestle, Microcentrifuge, Cooling centrifuge (12000 rpm), Vortex mixer, Waterbath

Sterile, DNAse free microcentrifuge tubes.

Reagents:

Ethanol 70%, Isopropanol

Extraction Buffer

20mM Tris HCL- pH:8.0
25mM EDTA – pH: 8.0
250mM NaCl
0.5% SDS

3.6.2. Lyse the tissue

Using a mortar and pestle, grinded up to 10mg of fresh gut sample in liquid nitrogen. Place the tissue sample in a clean, sterile microcentrifuge tube. To the tissue sample, 300µl of Cell Lysis Buffer was added. Thoroughly homogenise the material with 30-

35 strokes using a tube pestle. Added 20µl of Proteinase K and incubated at 58°C for 1 hour to overnight, until lysis was completed and the mixture becomes clear. Added 10µl of RNase A and incubated for 5 minutes at 37°C. After cooling the lysate in room temperature, 300µl of lysate was the distributed in clean, sterile microcentrifuge tubes.

3.6.3. Precipitate the gDNA

150µl of precipitate was added to 300µl of sample lysate and the suspension was vortexed for 20 seconds. Centrifuge the suspension at 12,000 rpm for 3 minutes and transfer the supernatant to a clean, sterile microcentrifuge tube. Add an equal amount of isopropanol was added and dissolved it by inverting the tube slowly. Again, the solution was centrifuged for 3 minutes at 12,000 rpm. The precipitated DNA thus formed was visible as a white pellet.

3.6.4. Wash the gDNA

The supernatant was removed and the tube was turned upside down over a sheet of absorbent paper towel to allow the residual liquid to drain for a few minutes. 1ml of 70% ethanol was added and washed the DNA pellet several times by inverting the tube. After centrifuging the sample for 1 minute at 12000 rpm, the supernatant was removed carefully.

3.6.5. Resuspend the gDNA

The sample was incubated for 10 minutes at 50-55°C to allow the excess ethanol to evaporate. Resuspend the DNA pellet in TE Buffer in an appropriate volume. To thoroughly resuspend the DNA, incubate the sample at room temperature overnight or at 65°C for up to an hour. The DNA should be dissolved fully in a clear, colourless solution.

If there are particles present. Repurify the impure gDNA if the A260/280 ratio is <1.7 or the DNA sample is contaminated by buffer, protein, or RNA. The purified DNA was stored at 4°C for immediate use and at -20°C in aliquots for long- term storage.

3.6.6. PCR

PCR was set up using the extracted DNA samples along with archaeal- bacterial specific primer set for V3- V4 region 16S amplification using HiPurA^{TM} 16S rRNA PCR kit.

Complete procedure of PCR was done in the biosafety cabinet and all products were thawed on 4°C ice prior to experiment.

Setting the PCR Reaction mixture:

- 10µl 2X PCR Taq Mixture was added in a PCR tube (provided in the kit)
- 2µl archaeal- bacterial specific primer mix was added.

16S rRNA Arch- F– 5'ACGGGGYGCAGCAGGCGCGA3'

16SrRNAArch- R- 5'GGACTACVSGGTATCTAAT 3'

- 2µl extracted DNA template was added to the tube.
- Molecular Biology Grade Water was added to make the PCR mix a final volume of 20µl.
- Centrifuge the PCR tube in Microcentrifuge for 10 seconds at 6000rpm.
- PCR tube was placed in the PCR Machine and set the programme as follows:

1. Initial Denaturation : 94°C for 5 minutes
2. Denaturation : 94°C for 30 seconds
3. Annealing : 56°C for 30 seconds
4. Extension : 72°C for 45 seconds
5. Final Extension : 72°C for 5 minutes
6. Hold : 4°C for ∞

The amplified PCR product has been kept at -20°C for storage. For analysis, the Amplified PCR product was resolved in the 1.2% Agarose Gel Electrophoresis.

3.6.7. Agarose Gel Electrophoresis

3µL of PCR product was resolved on 1.2% agarose gel electrophoresis at 120V for 60 min.

0.60gram agarose in 50 ml TAE buffer was added and heated to 55 to 60° C. 0.5µl of Cybr safe green stain was mixed with the gel solution and poured into the sealed gel casting tray. Allowed the gel to set for 30 minutes at room temperature and comb was placed. The comb was removed after the gel was solidified. 2 µl of 5X gel loading buffer, 10 µl of DNA sample and 3µl of DNA ladder of 1kb was added. The gel was run at 120V for 60 minutes. Agarose gel image was visuailised using UV Transilluminator (Biobee® Tech).

3.6.8. Fluorometer/ Nanodrop

The quantitative analysis of amplified genome was done in Qubit™ Fluorometer using Qubit™ Assay Kit.

Procedure:

1. Two assay tubes were arranged for the standards, and one assay tube was designated for each sample.
2. The Qubit™ working solution was created by diluting the Qubit™ reagent at a ratio of 1:200 in Qubit™ buffer.
3. Two hundred microliters of the working solution were dispensed for each sample and standard.
4. The assay tubes were prepared as follows:

Table 3.3. Fluorometer assay tubes preparation protocol

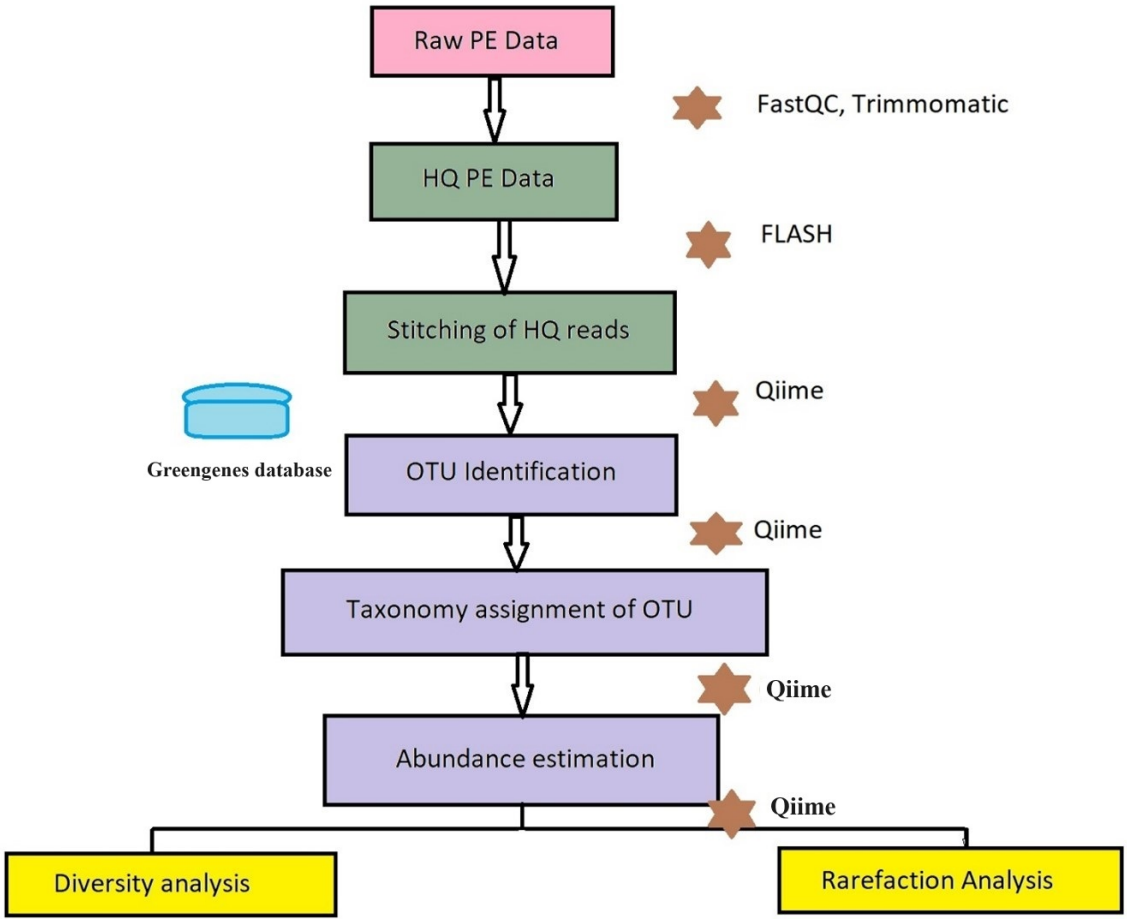
	Standard Assay tubes	Sample assay tubes
Volume of working solution	190µl	180-199µl
Volume of Standard	10µl	-
Volume of Sample	-	1-20µl
Total volume in each tube	200µl	200µl

5. All tubes were briefly vortexed for 2-3 seconds.
6. Subsequently, the tubes were set aside for incubation at room temperature for 2 minutes.
7. Readings for both standards and samples were then obtained using the Fluorometer.

3.6.9. Bioinformatic Analysis

A complete package of software called QIIME includes tools and methods including FastTree for heuristic-based maximum likelihood phylogeny inference (Price *et al.,* 2010), the RDP classifier for assigning taxonomic data using a naive Bayesian classifier (Wang *et al.,* 2007), and others. This enables QIIME, which is still being developed, to reasonably quickly and readily incorporate innovative independent tools and therefore advance in line with developments in the area of microbial community ecology.

Figure 3.7. Workflow of Bioinformatic Analysis



The following actions were taken to process the data from one 16S sample:

- High-quality, clean reads were obtained by employing Trimmomatic v0.38 (Bolger *et al*., 2014) with a sliding window of 10 bp and a minimum length of 100 bp. This process aimed to eliminate adapter sequences, ambiguous reads (reads with unknown nucleotides "N" greater than 5%), and low-quality sequences (reads with a phred score of less than 20 for more than 10% of the sequence).
- The raw data were stored in FastQ format (Andrews, 2010).
- Subsequently, paired-end (PE) data were merged into single-end reads using FLASH software (Magoˇc and Salzberg, 2011).
- Each operational taxonomic unit (OTU) was selected by comparing sample sequences against the Greengenes database (version 13_8) (McDonald *et al*., 2012), and OTUs were assigned based on sequence similarity within the readings.
- Reference databases were utilized to associate the OTUs with taxonomic identifications.
- Diversity metrics were calculated for each sample, and community types were compared using QIIME (Caporaso *et al*., 2010).

OTU-picking reveals highly comparable sequences in the sample and offers a comparative tool for community structure. The sequences from the samples were grouped into operational taxonomic units (OTUs) based on their degree of sequence similarity. OTUs are collections of sequences that are usually created using UNCLUST at 97% sequence similarity in order to reflect some degree of taxonomic relatedness. Each resultant cluster typically denotes a species. A sample sequence for each OTU is chosen for downstream analysis since each OTU may consist of several sequences. The representative sequence had been utilised to identify the OTU's taxonomic group. This is the outcome of the many charts and figures at various taxonomic levels.

3.6.10. Rarefaction curve

Rarefaction involves the creation of rarefaction curves, allowing for the estimation of species richness for a specific number of individual samples. The graph depicts the

number of species as a function of the sample number. A steep slope on the left side suggests that there is still much to be discovered about the diversity of species. The horizontal axis represents the number of sequences considered in diversity computations, while the vertical axis represents the community's diversity.

3.7. MORPHOMETRIC PARAMETERS OF SILKWORM

3.7.1. Total Body Length

Periodic increases in the length of fifth instar larvae were noted in both the control and experimental groups. The average length (in centimeters) of ten randomly selected larvae from each group was manually measured using a scale and graph paper during the study.

3.7.2. Total Body Weight

Periodic increases in the weight of fifth instar larvae were observed in both the control and experimental groups. The average weight (in grams) was measured using an electronic balance (Shimatzu Electronic balance).

3.8. DISSECTION OF SILKWORM GUT

The healthy fifth instar larvae of silkworm *B. mori* from four groups were used for the experiment. Larvae were randomly selected from each group, dissected under sterile conditions, and their guts were rinsed with a normal saline solution (0.75% NaCl) before being stored at −20°C. The larval guts were homogenized in ice-cold saline using a mortar and pestle, followed by centrifugation. After two centrifugation cycles at 12,000 rpm for 15 minutes each, the resulting supernatants were collected for proteomic and digestive enzyme activities (Vitthalrao and Sucheta, 2012). The remaining healthy larvae from both groups were maintained to produce cocoons for morphometric and quality evaluation.

3.9. BIOCHEMICAL PARAMETERS

3.9.1. Estimation of protein

Protein concentration of larval gut was done by Lowry's method (Lowry *et al*., 1951)

Reagents use:

1. 2% Sodium Carbonate (Na_2CO_3) in 0.1 N Sodium Hydroxide (NaOH)
2. 1% Sodium Potassium (Na-K) Tartrate in H_2O

3. 0.5% Copper Sulphate ($CuSO_4.5\ H_2O$) in H_2O
4. Reagent I: 48 ml of A, 1 ml of B, 1 ml of C
5. Reagent II- 1-part Folin-Phenol (2 N): 1 part water
6. BSA Standard - 10 mg BSA dissolved in 100 ml of distilled water

Procedure:

0.2 ml to 1 ml of BSA working standard solution was taken in 5 test tubes .20 µl of the extracted gut sample was taken in a test tube and make up to 1 ml with distilled water. 4.5ml of reagent I was added and kept for 10 minutes. After the incubation 0.5 ml of reagent II (Folin's reagent) was added and incubated for 30 minutes. The optical density of the sample was measured in UV spectrophotometer at 670nm. 0.1 N NaOH serves as blank.

3.9.2. Estimation of digestive enzymes

3.9.2. i. α- Amylase enzyme assay

α- Amylase assay was determined by the method described as Miller, (1959). The activity of enzyme was determined based on the reduction in blue colour intensity results from enzyme hydrolysis of starch using 3, 5-dinitrosalicylic acid (DNSA) reagent.

Reagents

1. 1 % starch
2. "Sodium phosphate buffer (0.2 M pH 8.0)"
3. DNSA - 0.5 N

10 g of 3, 5-dinitrosalicylic acid was gradually added to in 700 ml of NaOH solution and dissolve thoroughly. 300 g of sodium potassium tartrate ($NaKC_4O_6.4H_2O$) was added and made a final volume of 1000ml by adding distilled water. A dark orange coloured solution was formed which is stable in room temperature for several days.

Protocol

1ml of 0.2M phosphate buffer was mixed with 2ml of 1% soluble starch solution. Then add 200 µl (2.5mg protein) of sample enzyme solution into the mixture and incubated for 30 minutes at 40 °C. Following the incubation, 2 mL of DNSA reagent was introduced to halt the reaction, and the mixture was subjected to a 10-minute

period in a boiling water bath. 5ml of distilled water was added after cooling the solution and the absorbance was measured in a spectrophotometer at 540 nm. Maltose serves as standard for measuring optical density. A unit is characterized as the quantity of enzyme needed to release one micro mole of reducing sugar per minute.

3.9.2. ii. Assay of lipase activity

The lipase activity was done by the method of Tsujita *et al*. (1989).

Reagents

1. ρ-nitrophenyl Acetate (50 mM)
2. Sodium phosphate buffer solution (1 M pH 7)

Protocol

1.72 ml of sodium phosphate buffer solution (1 M pH 7) was added to 180 µl ρ-nitrophenyl acetate (50 mM) as a substrate. 100 µl of sample enzyme solution was mixed and then incubated for 15 min at 37°C. The optical density was read at 405 nm in spectrophotometer. One unit of enzyme releases 1.0 µmoles of p-nitrophenyl per minute at pH 7.0, at 37°C using p-nitrophenyl acetate as the substrate.

3.9.2. iii. Assay of protease activity

Protease activity was determined using azocasein as the substrate by Garcia-Carreno and Haard, (1993) method.

Reagents

1. 2% casein in Sodium phosphate buffer (40 mM pH 7.2)
2. Sodium phosphate buffer (40 mM pH 7.2)
3. 10 % Trichloro acetic acid (TCA)
4. NaOH (1 N)

Protocol

80 µl of 2% casein solution was added into 40 mM sodium phosphate buffer (pH 7.2). A 30 µL enzyme solution was combined with the reaction mixture and allowed to incubate for 60 minutes at 37°C. To arrest the proteolysis, 300 µL of 10% TCA was introduced into the mixture. The resulting precipitate was cooled at 4°C for 120

minutes, and the reaction mixture was then centrifuged at 10,000 rpm for 10 minutes. An equivalent volume of 1 N NaOH was added to the supernatant, and the absorbance was measured at 440 nm.

3.9.2. iv. Assay of cellulase activity

Cellulolytic enzyme activity was determined as described by Ghose (1987) method.

Reagents

1. Whatman No. 1 filter paper
2. 50 mM sodium acetate buffer (pH 4.8)
3. 3, 5- dinitrosalicylic acid (DNSA)

Protocol

50 mg of Whatmann no.1 filter paper was used for the filter paper cellulase activity assay. Mixing 1 mL of enzyme solution with 2 mL of 50 mM sodium acetate buffer (pH 4.8), the resulting mixture was incubated for 60 minutes at 50°C. To halt the enzyme reaction at the conclusion of the incubation period, 3 mL of DNSA reagent was added. The absorbance of enzyme was measured by UV- VIS spectrophotometer at 540nm against reagent blank and glucose solution served as standard.

3.10. MOLECULAR PARAMETERS

The dissected gut sample of five larvae from each treatment group was collected and pooled in eppendorf tubes containing trizol reagent and stored at -20°C.

3.10.1. mRNA isolation

Highly purified mRNA from the gut sample was isolated using Aurum Total RNA mini kit (Biorad) according to the manufacture's protocol.

1. 10mg of tissue samples from each group were properly weighed and homogenised in liquid nitrogen with a mortar and pestle. The release of genomic DNA into the solution causes the lysate to become highly dense after lysis. The viscosity of the lysate was lowered by homogenization with a REMI lab stirrer homogenizer.

2. After 3 minutes, centrifuge the lysate and transfer the supernatant to a fresh 2.0 ml capped microcentrifuge tube.
3. A homogenizer was used to completely mix 700µl of 60% ethanol into the supernatant.
4. The resulting suspension was passed through the RNA binding column provided with the kit and centrifuged for 60 seconds at 13,000 rpm.
5. The RNA binding column was rinsed with 700µl of low stringency solution and centrifuged for 30 seconds at 13,000 rpm.
6. Removed the column and discarded the low stringency wash solution from the was tube.
7. It added 80µl of the given diluted DNAse I to the membrane stack at the bottom of each column.
8. Incubated the digest at room temperature for 25 minutes. This procedure will eliminate genomic DNA.
9. The RNA binding column was washed twice, once with a high stringency wash solution and once with a low stringency wash solution.
10. Following the washes, the column was centrifuged for two minutes to remove the leftover wash solution.
11. Transfer the RNA binding column to a fresh 1.5ml capped microcentrifuge tube and add 80µl of the eluted solution onto the membrane stack at the bottom of the RNA binding column.
12. This was left undisturbed for one minute to allow the solution to saturate the membrane.
13. Following the incubation, the sample was centrifuged for two minutes to elute the total RNA.
14. By loading 2l of the sample, the RNA quality was tested using Agarose Gel Electrophoresis.
15. The retrieved RNA was quantified at 260nm, and purity was determined at 260/280nm.

3.10.2. cDNA preparation

cDNA was synthesised from extracted mRNA using the Biorad iScript cDNA synthesis Kit. The package included an unusual combination of oligo (dt) and random

hexamer primers. The reaction was carried out by incubating the reaction mixture in a thermal cycler according to the instructions protocol.

3.10.3. Preparation of reaction mixture

Table 3.4. Preparation of reaction mixture

Component	Volume per reaction µl
5x iScript Reaction Mixture	**4**
iScript reverse transcriptase	**1**
Nuclease free water	**Variable**
RNA template (100fg- 1 µg total RNA)	**Variable**
Total volume	**20**

3.10.4. Reaction protocol

Incubate the complete reaction in a thermal cycler using the following protocol:

Priming : 5minutes at 25°C

Reverse transcription : 20 minutes at 46°C

RT inactivation : 1 minute at 95°C

Optional step : Hold at 4°C

The resulting cDNA has been preserved for future use in PCR or RT. RNA was obtained from the samples using the Aurum Total RNA small kit, as directed by the manufacturer's instructions. 5µl of each sample was loaded into a 1% agarose gel from the extracted RNA samples. The image was captured with Gelstan 4X Advanced software and the Gel_i_ink gel doc system.

3.10.5. cDNA conversion

Following the previous technique, the obtained total RNA was utilised to synthesise cDNA. 2ul of cDNA was extracted from each sample and used for real-time PCR analysis.

3.10.6. Real time PCR Analysis

A comparative CT (ΔΔCT) real time PCR was used to evaluate gene expression using a biorad CFX connect RT PCR equipment and iTaq Universal SYBR Green Supermix.

3.10.7. Real time PCR Mix

cDNA	:	2µl
Primer F	:	2 picomoles (Final con) - 1 µl
Primer R	:	2 picomoles (Final con) - 1 µl
SYBR green mix	:	10 µl (Final 1x)
Water	:	4 µl
Total volume	:	20 µl

The volume of cDNA could fluctuate based on the initial amount of mRNA.

3.10.8. List of Primers

The following primers are used for mRNA expression analysis.

Table 3.5. List of Primers

Genes	cDNA accession number	5' – 3' primer sequence	Primer annealing	Product size
Hsp 70	DQ311189.1	FP-GAACACACTCGCTGCACATC	58°C	400bp
		RP-GAGGAGTGCCCAAGATCGAC		
Hsp 40	AB206400	FP-TCGGACGATGACATCAAGAA	54°C	520bp
		RP-CCCGGGCGATATCTTCTAAT		
Hsp 20.4	AF315318	FP-TTTTGGCCTTGCCTTAACAC	57°C	453bp
		RP-TTCGCTCTGGTCCTTGATCT		
Hsp 90	NM_001043411.1	FP-AGGCCTTCGAACTTGTATCG	54.7°C	103bp
		RP-ATGGCAAGACCCTTGTATCT		

3.11. HISTOPATHOLOGY OF SILKWORM GUT

The gut samples were dissected out from the control and experimental groups and were fixed in Bouins fluid for histopathological analysis. The histological procedure was done by routine paraffin embedding technique. The tissue is dehydrated using a graded series of alcohol solutions with increasing concentrations after being fixed in a

formalin solution to maintain its structural integrity. After being dehydrated, the tissue is transparently cleaned with xylene and then embedded in paraffin wax. Following embedding, a section that was eight microns thick was sliced utilizing a microtome (Leica RM 2125 RT). The section was then stained with haematoxylin and eosin to highlight structural and cellular characteristics. Pathological changes manifested in the sections were observed and documented using a light binocular microscope (Leica ICC50 E) (Sun *et al*., 2022).

3.12. ECONOMIC PARAMETERS OF COCOON

Raw silk can only be obtained from cocoon shells. As a result, it is the most important economic element. All economic parameters were estimated in accordance with the normal approach (FAO Manual). Matured larvae were manually picked and placed to mountages for cocooning on the fifth day of the fifth instar. The seed cocoons were removed on the eighth day of spinning after the cocoons formed within 72 hours of mounting. Cocoons were kept at a temperature of 25°C and a humidity of 75%. Quality and quantity evaluation of cocoon yield were calculated.

3.12.1. Average single cocoon weight: 20 cocoons were randomly selected from each group on 6^{th} day of spinning were weighed and calculated the average.

3.12.2. Percent good cocoons: Unsized and damages cocoons were removed from the group and percentage of good cocoon yield were calculated as follows.

$$Percentage\ of\ good\ cocoons = \frac{number\ of\ good\ cocoons\ (reelable)}{total\ number\ of\ harvested\ cocoons} \times 100$$

3.12.3. Cocoon shell weight: 20 cocoons were randomly selected and shells were weighed after removing the pupae by using electronic balance.

3.12.4. Cocoon shell percentage (CSP)

$$CSP = \frac{cocoon\ shell\ weight}{cocoon\ weight} \times 100$$

3.12.5. Filament length: The filament length was measured by an epprovette and recorded accordingly in cm.

3.12.6. Filament weight: The cocoon was reeled in reeling machine and the weight of the single filament was measured in grams (g).

3.12.7. Filament Size: The size of the filament was calculated by following formula.

$$Denier = \frac{weight\ of\ the\ filament}{length\ of\ the\ filame} \times 9000$$

3.12.8. Reelability percentage: It shows the percentage of reeling capacity of a cocoon.

$$Reelability\ \% = \frac{weight\ of\ the\ cocoon\ filament}{weight\ of\ the\ cocoon} \times 100$$

3.12.9. Tenacity and Elongation: The instrument VIBRODYN 500 was used for measuring the tenacity and elongation properties of the silk filament. This instrument utilizes dynamic testing methods to assess the strength and stretch characteristics of the material. The fiber samples are subjected to controlled tension and elongation, allowing for the calculation of tenacity (the maximum force the fiber can withstand per unit cross-sectional area) and elongation (the extent to which the fiber can deform before breaking). Testing is carried out as per ASTM D 3822 - 14 (2020) for Fibre Tenacity and elongation.

3.13. STATISTICAL ANALYSIS

The statistical tool R programming version 4.0.3 was used to analyse the data in statistical terms. Results were presented as mean standard error of six replicates. A one-way analysis of variance (ANOVA) and Tukey's test were conducted. At $P \leq 0.05$, the results were considered significant in each case.

CHAPTER 4

RESULTS

4. Results

4.1. GUT BACTERIAL COMPOSITION AND DIVERSITY

4.1.1. Culture dependent method

Three different bacterial colonies were isolated from a single group and a total of twelve colonies were isolated from the four groups based on their shape and colony colour from the agar plates (**Fig 4.1**). Then isolated colonies were further screened by gram reaction and biochemical characteristics. Bacillus is the common bacteria identified from the four groups. Citrobacter, Pseudomonas, Enterobacter, Lactobacillus and Klebsiella were the other bacteria identified from the groups.

4.1.2. Bacterial isolates of Control (CN)

The bacterial isolates present in the gut tissues of the control group (CN) were Bacillus, Citrobacter and Pseudomonas (**Table 4.2**). Citrobacter and Pseudomonas were the Gram-negative rods; Bacillus was the Gram-positive rod -shaped bacteria. Citrobacter and pseudomonas belong to the phylum proteobacteria and bacillus belong to phylum firmicutes.

4.1.3. Bacterial isolates of Thermal Stress Group (T1)

The bacterial isolates present in the gut tissues of thermal Stress Group (T1) were Enterobacter, Bacillus and Klebsiella (**Table 4.3**). Enterobacteria and Klebsiella were the gram-negative rods belong to the phylum proteobacteria and bacillus was the gram positive bacteria belonging to the phylum firmicutes.

4.1.4. Bacterial isolates of Vitamin C supplemented Group (T2)

The bacterial isolates present in the gut tissues of vitamin C supplemented Group (T2) were Pseudomonas, Bacillus and Lactobacillus (**Table 4.4**). The bacterial Bacillus and Lactobacillus were gram-positive rod-shaped bacteria from the phylum firmicutes, whereas Pseudomonas was gram-negative rod bacteria which belongs to the phylum proteobacteria.

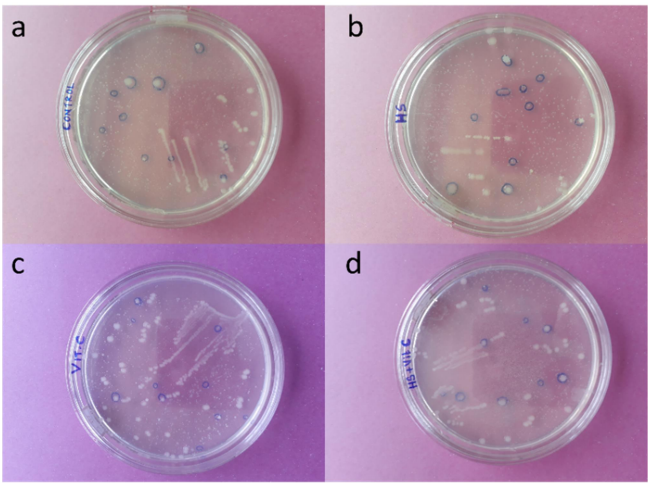
4.1.5. Bacterial isolates of Thermal Stress + Vit C supplemented Group (T3)

The bacterial isolates present in the gut tissues of thermal stress + vit C supplemented Group (T3) were Klebsiella, Bacillus and Pseudomonas (**Table 4.5**). Klebsiella and pseudomonas were gram negative bacteria belongs to the phylum Proteobacteria and bacillus is gram positive bacteria belongs to the phylum Firmicutes.

Table 4.1: Bacterial colony isolates and their codes of control and experimental groups.

Sample collected	Isolates	Code
Control (CN)	3	CN1, CN2, CN3
Thermal Stress (T1)	3	T11, T12, T13
Vitamin C supplemented (T2)	3	T21, T22, T23
Vit C+ Thermal stress (T3)	3	T31, T32, T33

Figure 4.1: Bacterial colonies of control and treated groups.



a: CN-Control, b: T1-Thermal stress, c: T2- Vit C Supplemented, d: T3-Thermal stress + Vit C Supplemented.

Figure 4.2: Bacterial isolates of control and treated groups.

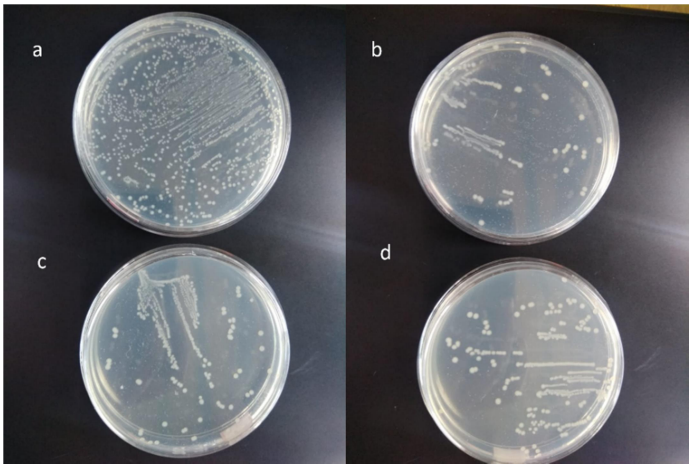


Table 4.2: Morphological characters of the microbial colonies of Control and experimental groups

Groups	Bacterial Isolates	Colony Colour	Colony Shape	Vegetative cell shape	Gram reaction	Aerobic/Anaerobic
CN	CN1	Whitish	Round	Rod	Positive	Aerobic
	CN2	Pale yellowish	Round	Rod	Negative	Aerobic
	CN3	Creamy white	Irregular	Rod	Negative	Aerobic
T1	T11	Grey	Round	Rod	Negative	Aerobic
	T12	Greyish white	Round	Rod	Negative	Aerobic
	T13	Whitish	Round	Rod	Positive	Aerobic
T2	T21	Creamy white	Irregular	Rod	Negative	Aerobic
	T22	Whitish	Round	Rod	Positive	Aerobic
	T23	Whitish	Round	Rod	Positive	Aerobic
T3	T31	Greyish white	Round	Rod	Negative	Aerobic
	T32	Whitish	Round	Rod	Positive	Aerobic
	T33	Creamy white	Irregular	Rod	Negative	Aerobic

Table 4.3: Biochemical characteristics of bacterial isolates of Control Group (CN)

Biochemical Test	**Isolate CN1**	**Isolate CN2**	**Isolate CN3**
Indole test	-	-	-
Methyl red test	+	+	-
Voges Proskauer test	-	-	-
Citrate test	+	+	+
Urease test	-	+	-
Catalase test	+	+	+
Oxidase test	-	-	+
H2S production test	-	+	-
Nitrate reduction test	+	+	+
Gelatin liquefaction test	+	-	+
Starch Hydrolysis test	+	+	-
Carbohydrate fermentation test	+	+	+
Bacterial Sp.	**Bacillus**	**Citrobacter**	**Pseudomonas**

Table 4.4: Biochemical characteristics of bacterial isolates of Thermal Stress Group (T1)

Biochemical Test	**Isolate T11**	**Isolate T12**	**Isolate T13**
Indole test	-	+	-
Methyl red test	-	+	+
Voges Proskauer test	+	-	-
Citrate test	+	-	+
Urease test	-	+	-
Catalase test	+	+	+
Oxidase test	-	-	-
H2S production test	-	+	-
Nitrate reduction test	+	+	+
Gelatin liquefaction test	-	+	+
Starch Hydrolysis test	+	+	+
Carbohydrate fermentation test	+	+	+
Bacterial Sp.	**Enterobacter**	**Klebsiella**	**Bacillus**

Table 4.5: Biochemical characteristics of bacterial isolates of Vit C supplemented Group (T2)

Biochemical Test	**Isolate T21**	**Isolate T22**	**Isolate T23**
Indole test	-	-	-
Methyl red test	-	+	-
Voges Proskauer test	-	-	+
Citrate test	+	+	-
Urease test	-	-	-
Catalase test	+	+	-
Oxidase test	+	-	-
H2S production test	-	-	-
Nitrate reduction test	+	+	-
Gelatin liquefaction test	+	+	-
Starch Hydrolysis test	-	+	+
Carbohydrate fermentation test	+	+	+
Bacterial Sp.	**Pseudomonas**	**Bacillus**	**Lactobacillus**

Table 4.6: Biochemical characteristics of bacterial isolates of Thermal Stress + Vit C supplemented Group (T3)

Biochemical Test	**Isolate T31**	**Isolate T32**	**Isolate T33**
Indole test	+	-	-
Methyl red test	+	+	-
Voges Proskauer test	-	-	-
Citrate test	-	+	+
Urease test	+	-	-
Catalase test	+	+	+
Oxidase test	-	-	+
H2S production test	+	-	-
Nitrate reduction test	+	+	+
Gelatin liquefaction test	+	+	+
Starch Hydrolysis test	+	+	-
Carbohydrate fermentation test	+	+	+
Bacterial Sp.	**Klebsiella**	**Bacillus**	**Pseudomonas**

4.1.6. Culture independent method – Metagenomic analysis of silkworm gut.

Metagenomic analysis of gut microbes of silkworms in control and treated groups were done to identify the diversity and composition of symbiotic bacteria present in the gut of silkworm under thermal treated conditions. Total 695,805 OTUs were obtained from the gut samples of silkworms in the control and treated groups in which 287251 were present in control group, 115895 in the group T1, 160773 in T2 and 131886 in T3 group (**Table 4.8**). When analysing the diversity of species level, it was found that 458 species reported in the control group whereas only 434 species were reported in the thermal stress exposed group [T1], 440 species were present in the group T3 and 436 species in the group T4. These results showed that the gut microflora of silkworm drastically decreased not only in their abundance but also in the species diversity when exposed to thermal stress. Comparative analysis of relative abundance of bacteria in control and experimental groups at the class level (Fig. 4.6), order level (Fig. 4.7), family level (Fig. 4.8) and genus level (Fig. 4.9) were presented.

A total of 695,805 operational taxonomic units (OTUs) were identified from silkworm gut samples in both the control and treated groups. Among these, 287,251 were found in the control group, 115,895 in group T1, 160,773 in T2, and 131,886 in T3 (Table 4.8). When examining species-level diversity, the control group exhibited 458 species, while the thermal stress-exposed groups (T1, T2, and T4) reported 434, 440, and 436 species, respectively. These findings indicate a significant decrease not only in the abundance but also in the species diversity of silkworm gut microflora when exposed to thermal stress. A comparative analysis of the relative abundance of bacteria in the control and experimental groups was conducted at various taxonomic levels, including class (Fig. 4.6), order (Fig. 4.7), family (Fig. 4.8), and genus (Fig. 4.9).

Table 4.7: Purity and quantity analysis of isolated gut genomic DNA from control and treated groups of B. mori

Sample	Nanodrop Readings (ng/µl)	Nanodrop OD A260/280	Nanodrop OD A260/230
CN	306.5	1.88	1.50
T1	380.5	1.90	1.82
T2	261.9	1.96	1.51
T3	407.1	1.87	1.04

Figure 4.3: The genomic DNA isolated from control and treated groups of *B. mori* gut were checked for purity in 1% agarose gel. 1 Kb ladder was used for genomic DNA.

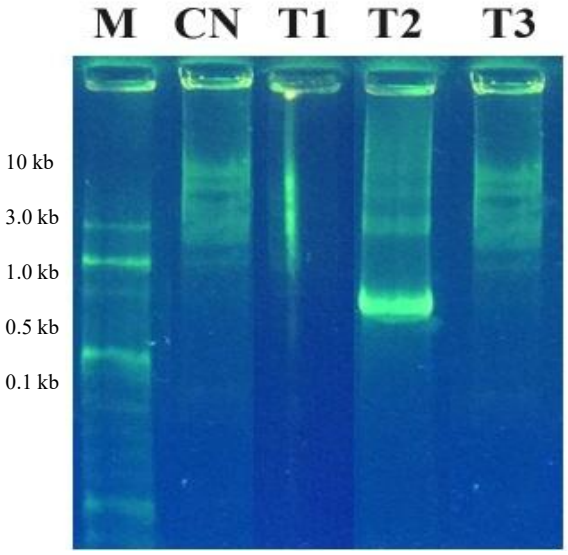


Figure 4.4: PCR amplified V3 – V4 products in 1.2 % agarose gel. 100 bp ladder was used for amplicon DNA quantification.

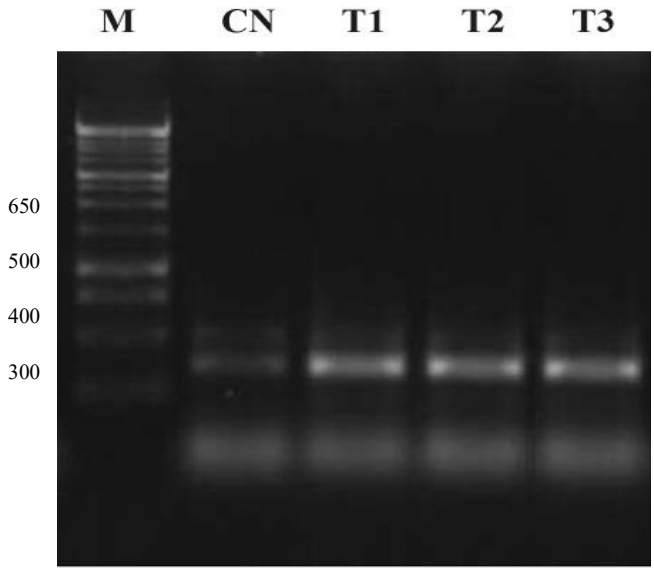


Table 4.8: Microbial composition of silkworm gut in the control (CN) and treated groups (T1, T2, T3).

Sample	No: of reads	Phylum	Class	Order	Family	Genus	Species
CN	287251	27	73	132	227	401	458
T1	115895	29	72	122	219	379	434
T2	160773	27	68	120	221	389	440
T3	131886	27	66	116	212	376	436

Figure 4.5: Abundance of gut bacteria in control and experimental groups.

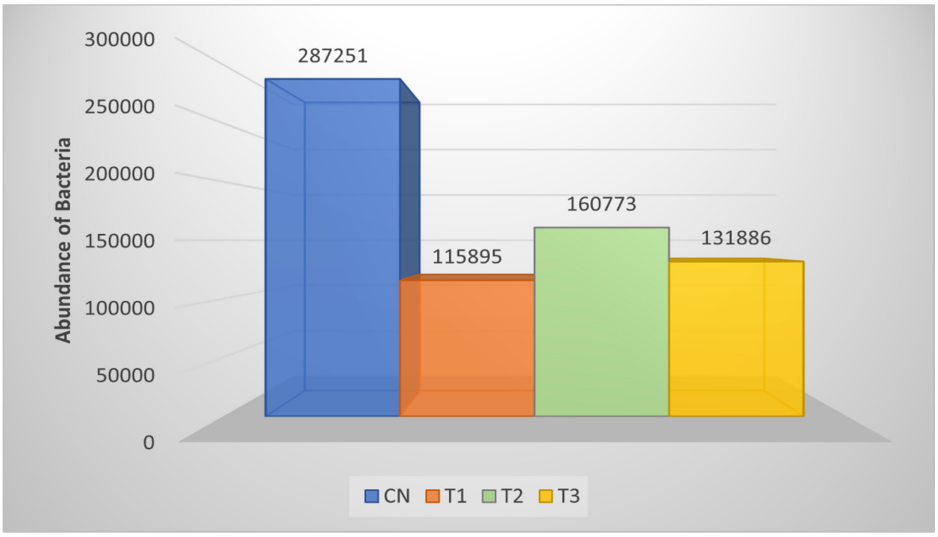
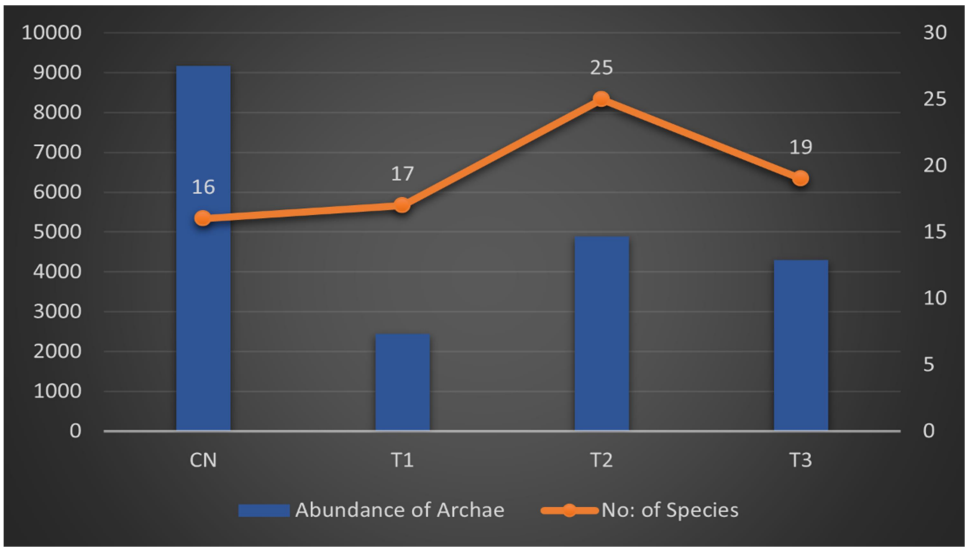


Figure 4.6: **Abundance and number of species of Archaea in control and treated groups.**

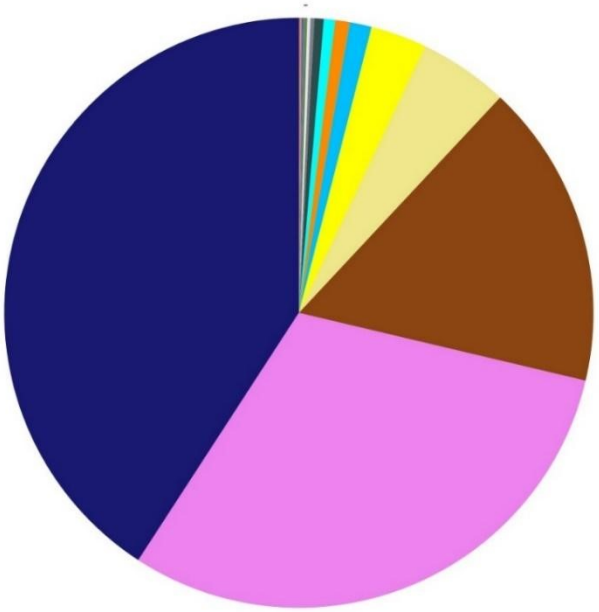


4.1.7. Phylum level analysis

At the phylum level analysis, Firmicutes, Bacteroidetes, and Proteobacteria emerged as the dominant bacteria acro4.19ss all groups. In the CN group, their relative abundance was 40.82%, 30.48%, and 16.72%, respectively (Fig 4.7a). For the T1 group, these values were 38.99%, 29.08%, and 21.63%, respectively (Fig. 4.7b). In the T2 group, the relative abundance was 37.72%, 31.67%, and 18.26%, respectively (Fig 4.7c), while for the T3 group, it was 38.05%, 32%, and 18.84%, respectively (Fig 4.7d).

Figure 4.7: Relative Abundance of the gut microbes at the Phylum level. a: CN-Control, b: T1-Thermal stress, c: T2- Vit C Supplemented, d: T3- Thermal stress + Vit C Supplemented.

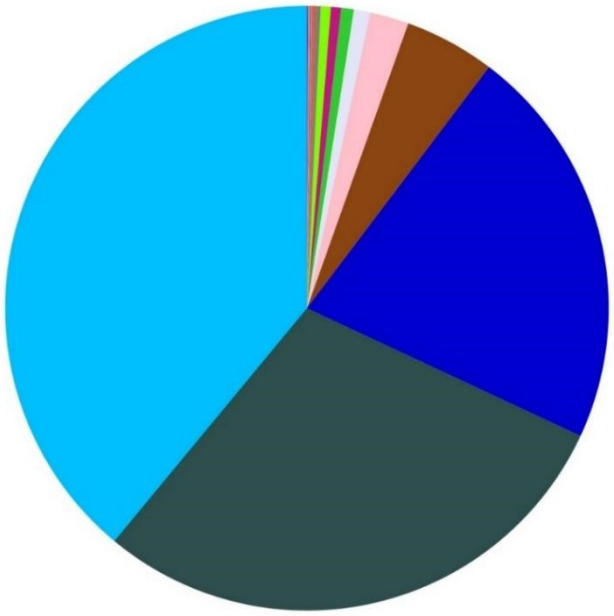
a)



CN Phylum legend

Legends	Taxonomy	Abundance
	k__Bacteria;p__Firmicutes	40.82%
	k__Bacteria;p__Bacteroidetes	30.48%
	k__Bacteria;p__Proteobacteria	16.72%
	k__Bacteria;p__Deferribacteres	4.92%
	k__Archaea;p__Euryarchaeota	3.08%
	k__Bacteria;p__Verrucomicrobia	1.2%
	k__Bacteria;p__Planctomycetes	0.8%
	k__Bacteria;p__Actinobacteria	0.62%
	k__Bacteria;p__Chloroflexi	0.51%
	k__Bacteria;p__TM7	0.21%
	k__Bacteria;p__Lentisphaerae	0.18%
	k__Bacteria;p__Acidobacteria	0.13%
	k__Archaea;p__Crenarchaeota	0.11%
	k__Bacteria;p__BRC1	0.05%
	k__Bacteria;p__Elusimicrobia	0.03%
	k__Bacteria;p__Fusobacteria	0.03%
	k__Bacteria;p__Chlorobi	0.02%
	k__Bacteria;p__Gemmatimonadetes	0.02%
	k__Bacteria;p__WS3	0.01%
	Others	0.05%

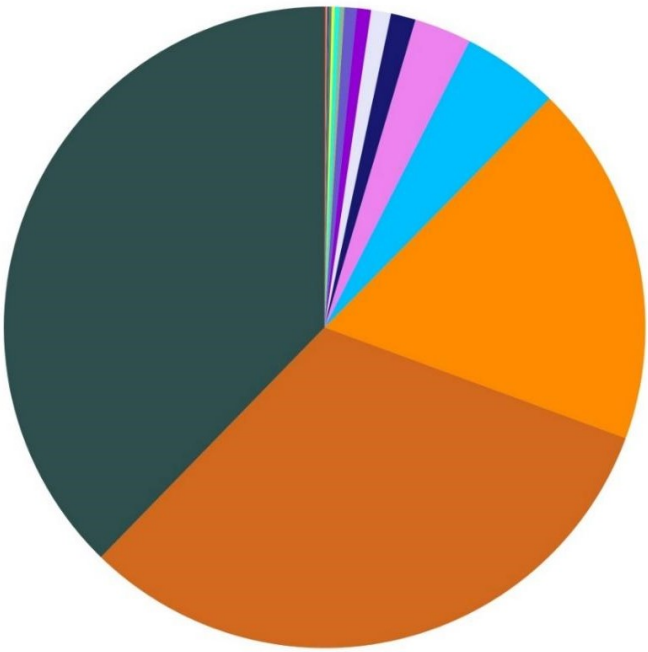
b)



T1 Phylum legend

Legends	Taxonomy	Abundance
	k__Bacteria;p__Firmicutes	38.99%
	k__Bacteria;p__Bacteroidetes	29.08%
	k__Bacteria;p__Proteobacteria	21.63%
	k__Bacteria;p__Deferribacteres	4.86%
	k__Archaea;p__Euryarchaeota	2.08%
	k__Bacteria;p__Verrucomicrobia	0.88%
	k__Bacteria;p__Actinobacteria	0.68%
	k__Bacteria;p__Planctomycetes	0.54%
	k__Bacteria;p__Chloroflexi	0.54%
	k__Bacteria;p__TM7	0.23%
	k__Bacteria;p__Lentisphaerae	0.13%
	k__Bacteria;p__Acidobacteria	0.11%
	k__Bacteria;p__Elusimicrobia	0.04%
	k__Bacteria;p__BRC1	0.04%
	k__Archaea;p__Crenarchaeota	0.04%
	k__Bacteria;p__Spirochaetes	0.03%
	k__Bacteria;p__WS3	0.03%
	k__Bacteria;p__Thermotogae	0.02%
	k__Bacteria;p__Fusobacteria	0.02%
	Others	0.04%

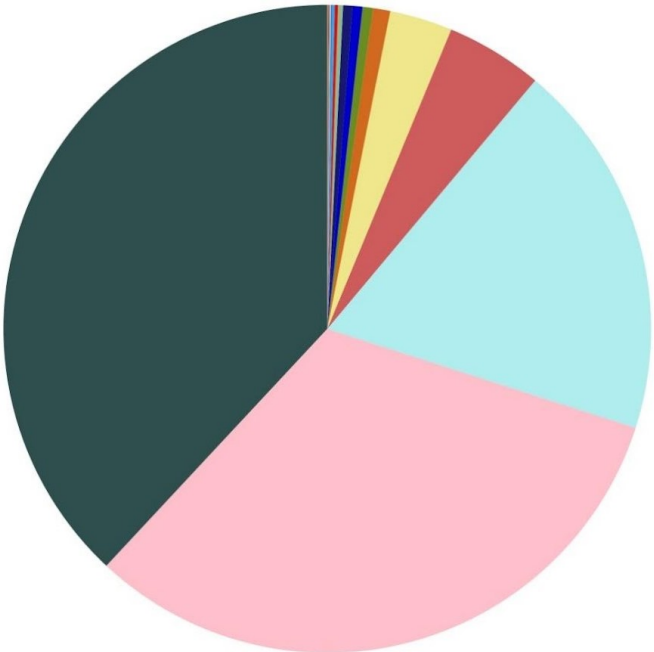
c)



T2 Phylum legend

Legends	Taxonomy	Abundance
	k__Bacteria;p__Firmicutes	37.72%
	k__Bacteria;p__Bacteroidetes	31.67%
	k__Bacteria;p__Proteobacteria	18.26%
	k__Bacteria;p__Deferribacteres	4.9%
	k__Archaea;p__Euryarchaeota	2.89%
	k__Bacteria;p__Verrucomicrobia	1.25%
	k__Bacteria;p__Planctomycetes	1.0%
	k__Bacteria;p__Chloroflexi	0.71%
	k__Bacteria;p__Actinobacteria	0.63%
	k__Bacteria;p__TM7	0.26%
	k__Bacteria;p__Lentisphaerae	0.22%
	k__Archaea;p__Crenarchaeota	0.15%
	k__Bacteria;p__Acidobacteria	0.13%
	k__Bacteria;p__BRC1	0.04%
	k__Bacteria;p__Thermotogae	0.03%
	k__Bacteria;p__Fusobacteria	0.03%
	k__Bacteria;p__WS3	0.03%
	k__Bacteria;p__Chlorobi	0.02%
	k__Bacteria;p__Tenericutes	0.02%
	Others	0.05%

d)



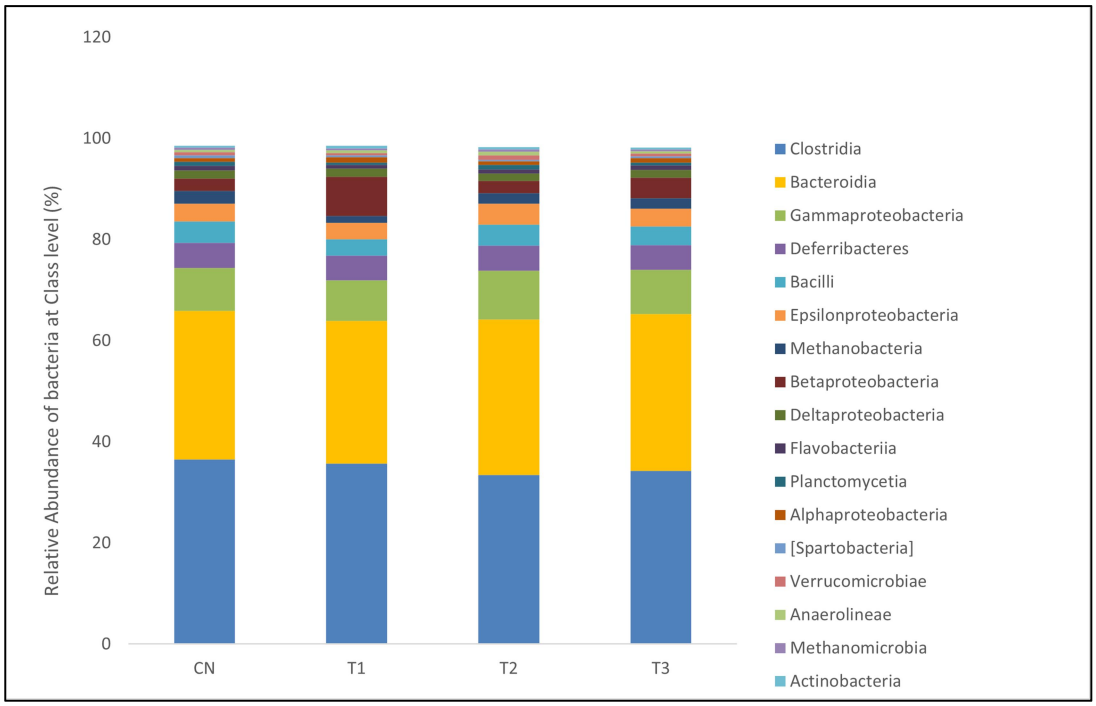
T3 Phylum legend

Legends	Taxonomy	Abundance
	k__Bacteria;p__Firmicutes	38.05%
	k__Bacteria;p__Bacteroidetes	32.0%
	k__Bacteria;p__Proteobacteria	18.84%
	k__Bacteria;p__Deferribacteres	4.85%
	k__Archaea;p__Euryarchaeota	3.13%
	k__Bacteria;p__Verrucomicrobia	0.86%
	k__Bacteria;p__Planctomycetes	0.52%
	k__Bacteria;p__Actinobacteria	0.49%
	k__Bacteria;p__Chloroflexi	0.47%
	k__Bacteria;p__TM7	0.25%
	k__Bacteria;p__Lentisphaerae	0.16%
	k__Archaea;p__Crenarchaeota	0.13%
	k__Bacteria;p__Acidobacteria	0.07%
	k__Bacteria;p__BRC1	0.05%
	k__Bacteria;p__Thermotogae	0.02%
	k__Bacteria;p__Spirochaetes	0.02%
	k__Bacteria;p__Gemmatimonadetes	0.02%
	k__Bacteria;p__Elusimicrobia	0.01%
	k__Bacteria;p__Nitrospirae	0.01%
	Others	0.04%

4.1.8. Class level analysis

The comparison of predominant taxa at class level analysis of CN, T1, T2 and T3 larval guts were clostridia (36.5%, 35.7%, 33.4% and 34.24%), bacteroidia (29.4%, 35.7%, 33.4% and 34.24%), gammaproteobacteria (8.46%, 7.9%, 9.6% and 8.75%), deferribacteres (4.91%, 4.8%, 4.89% and 4.84%) and bacilli (4.27%, 3.22%, 4.21% and 3.7%) respectively.

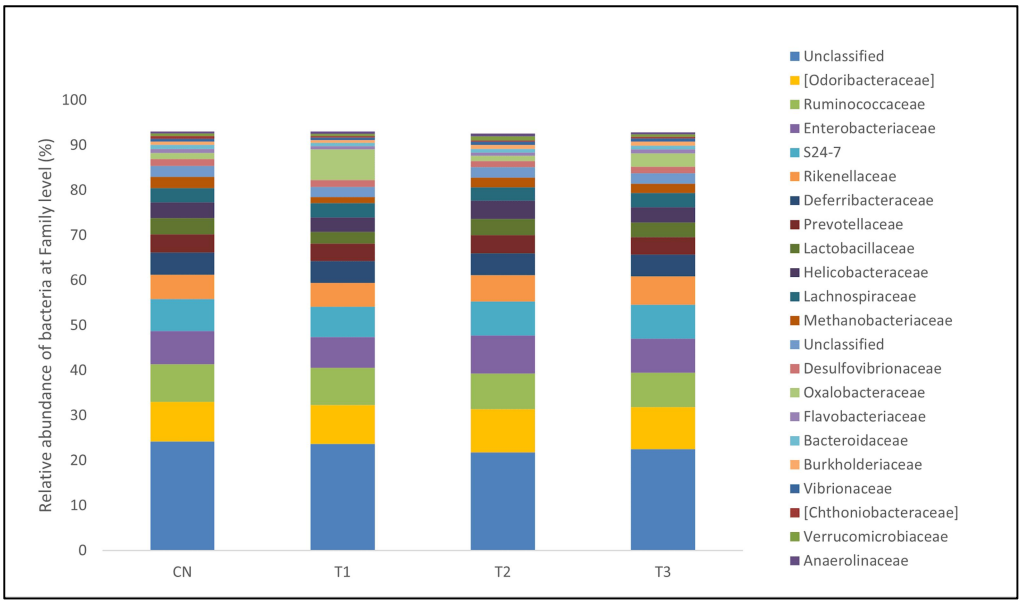
Figure 4. 8: **Relative abundance of bacteria at class level**



4.1.9. Family level analysis

Comparative analysis of the abundance of taxa at family level of CN, T1, T2 and T3 were Odoribacterracea (8.7%, 8.6%, 9.5% and 9.2%), Ruminococcaceae (8.39%, 8.2%, 7.9% and 7.6), Enterobacteriacea (7.29%, 6.8%, 8.4% and 7.5%) respectively. The majority of the taxa belongs to unclassified group (24.21%, 23.64%, 21.81% and 22.54 %).

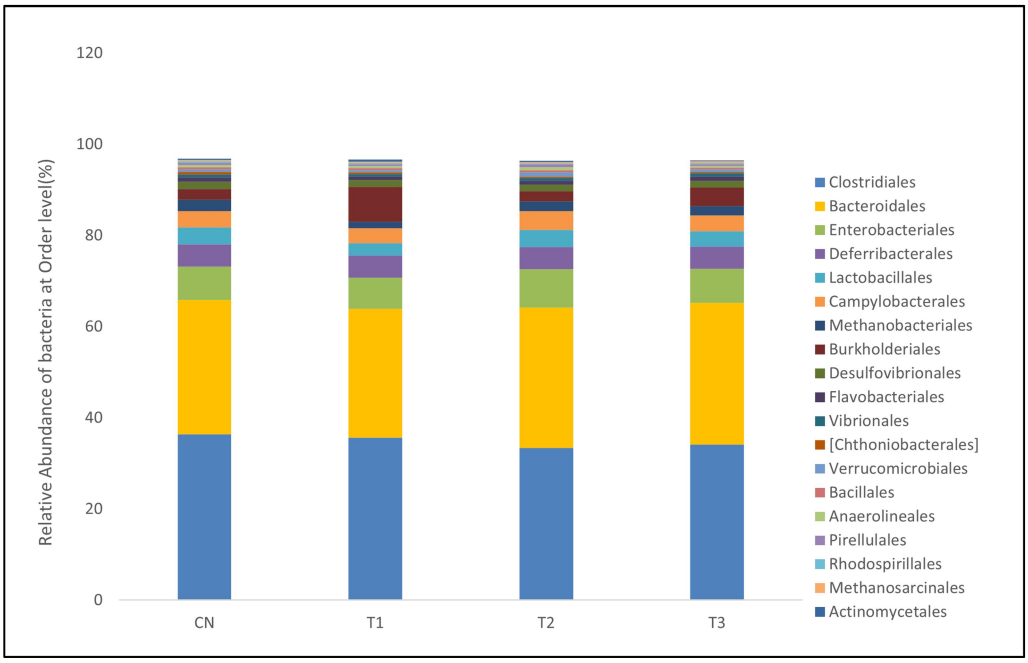
Figure 4.9: Relative abundance of bacteria at family level



4.1.10. Order level analysis

At the order level analysis, the dominant bacterial taxa in the control and treated groups (CN, T1, T2 and T3) were clostridiales (36.4%, 35.6%, 33.4% and 34.16%), bacteroidales (29.4%, 28.24%, 30.76% and 31%), enterobacteriales (7.2%, 6.8%, 8.4% and 7.5%), defferibacterales (4.9%, 4.8%, 4.89% and 4.84%) and lactobacillales (3.75%, 2.71%, 3.75% and 3.37%) respectively.

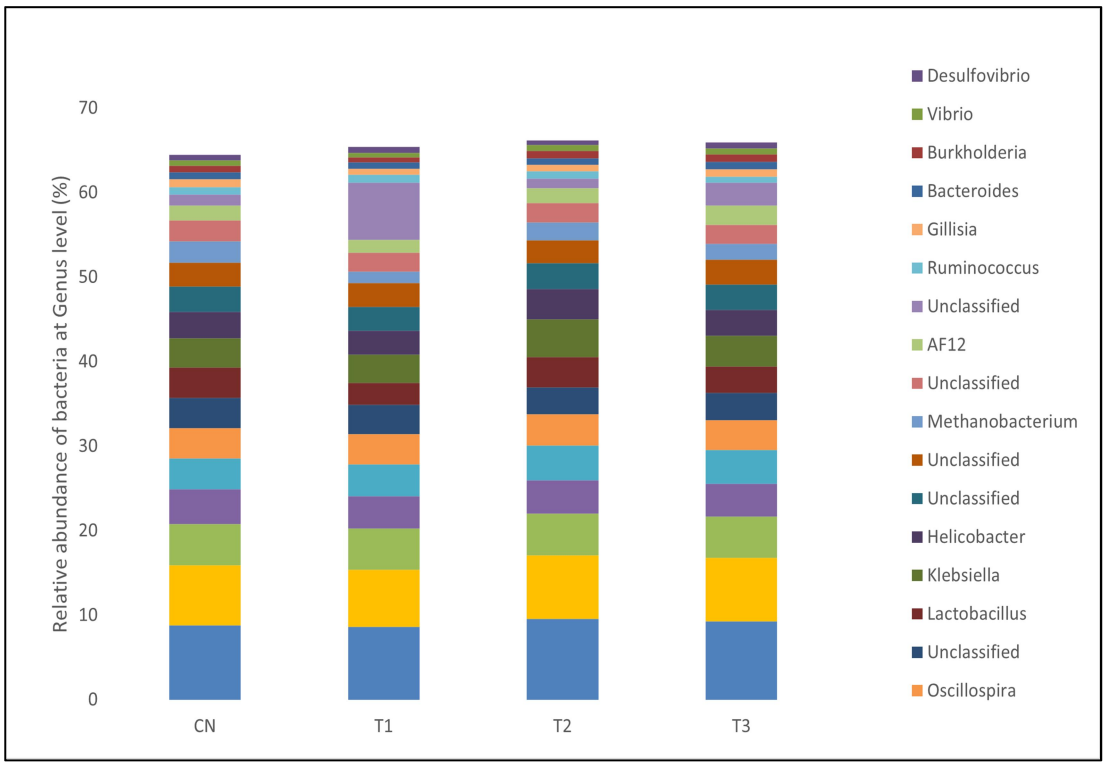
Figure 4.10: **Relative abundance of bacteria at order level**



4.1.11. Genus level analysis

At the genus level analysis, the relative abundance of gut microbes was lower in the thermal stress group compared to the control. The dominant bacteria in all four groups (CN, T1, T2, and T3) included Odoribacter (8.78%, 8.64%, 9.5%, 9.2%), Mucispirillum (4.9%, 4.8%, 4.89%, 4.84%), Prevotella (4.07%, 3.83%, 3.9%, 3.85%), Oscillopira (3.61%, 3.56%, 3.69%, 3.49%), Klebsiella (3.47%, 3.4%, 4.49%, 3.6%), and Lactobacillus (3.57%, 2.57%, 3.6%, 3.15%) respectively. Additionally, 24.21% of CN, 23.64% of T1, 22.5% of T2, and 21.8% of T3 gut microbes were unclassified.

Figure 4.11: **Relative abundance of bacteria at genus level**



4.1.12. Unique Species present in the gut of *B. mori* of CN, T1, T2 and T3 groups

It was observed that 59 species were unique to the control group, 38 unique species in the group T1, 40 unique species in group T2 and 37 unique species in Group T3. Some of the unique gut bacterial species of control and experimental groups of silkworm larvae were presented in Table 4.9.

Table 4.9: Unique Species present in the gut of *B. mori* of CN, T1, T2 and T3 groups

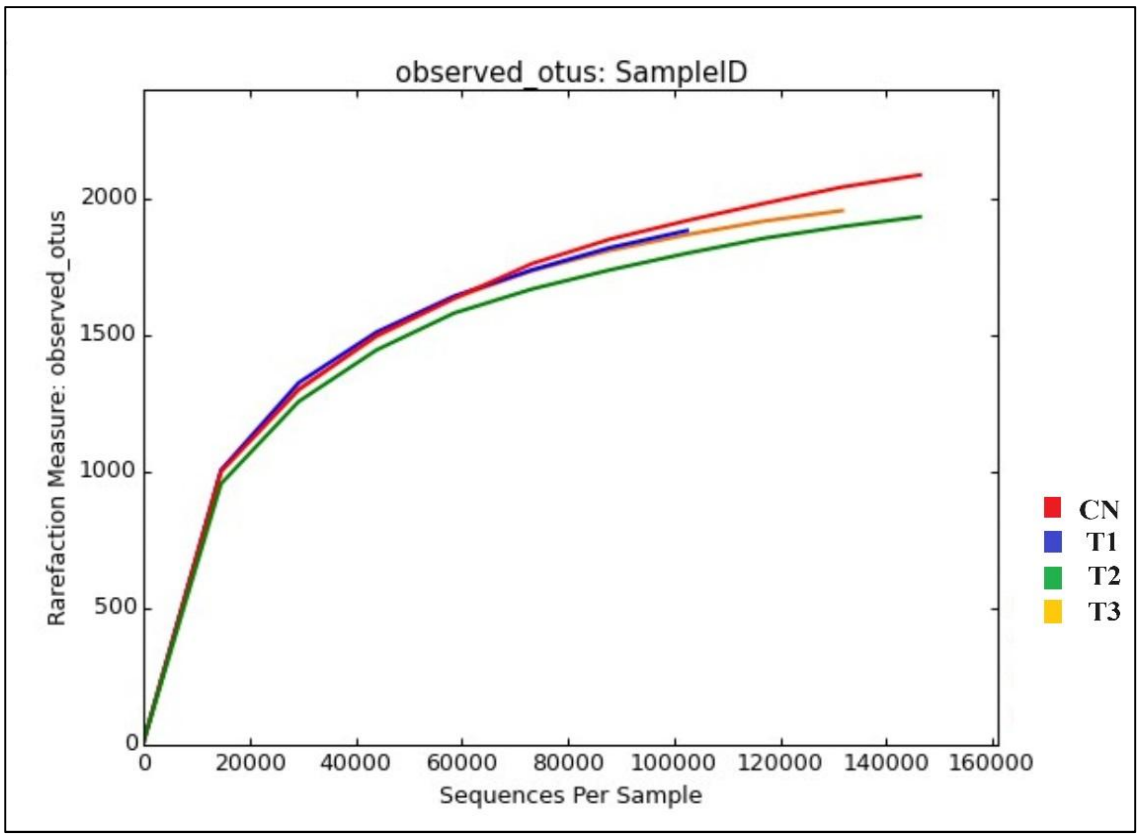
CN	T1	T2	T3
Bacillus acidicola	*Novosphinobium stygium*	*Prosthecobacter debontii*	*Methylobacterium mesophilicum*
Clostridium perfinges	*Blautia obeum*	*Alcanivorax dieselolei*	*Acidomonas methanolica*
Maribius salinus	*Paenibaillus hodogayensis*	*Constrictibacter antarticus*	*Hyphomicrobiyum zavarzinii*
Pseudonocardia halophotica	*Dietzia timorensis*	*Nitrosovibrio tenuis*	*Symbiobacterium thermophilum*
Streptococcus luteciae	*Planctomycete DWL312*	*Cetobacterium somerae*	*Clostridium neonatale*
Thermobacillus composti	*Halorhodospira*	*Clostridium diffcile*	*Bacteroides caccae*
Aquomicrobium aerolatum	*Marinobacter*	*Salirhabdus euzebyi*	*Verrucosispora gifhornensis*
Lactobacillus vaginalis	*Giesbergeria*	*Streptomyces mirabilis*	*Lapillicoccus jejuensis*
Lactobacillus zeae	*Allloicoccus*	*Streptomyces ahygroscopicus*	*Cenarchaeum symbiosium*
Flexispira rappini	*Modestobacter*	*Halostagnicola larsenii*	*Candidatus nitrososphaera*
Halogranum	*Haloterrigena*	*Methanoculleus*	*Thermacetogenium*

4.1.13. Rarefaction analysis of control and treated gut of *B. mori*

By generating the rarefaction curves, the degree of coverage of bacterial diversity associated to control and treated gut tissues was confirmed. As a function of the number of sequences collected, the curve plots show the total number of unique species annotations. The rarefaction plots are used as the basis for computing the diversity measures. The annotated species richness in the V3–V4 area was displayed on the rarefaction plot. The flattening of curves towards the right side of the graph

indicates that a sufficient number of sequences were sampled to provide adequate coverage of the gut bacteria (Fig 4.12). The rarefaction curve clearly indicated that heat shock had an impact on the diversity and richness of silkworm gut microbes. A noticeable reduction in both the number of species and the overall diversity of gut microbes was observed in the heat shock group when compared to the control group.

Fig. 4.12: Rarefaction analysis curve of gut of the silkworm in control and experimental groups.

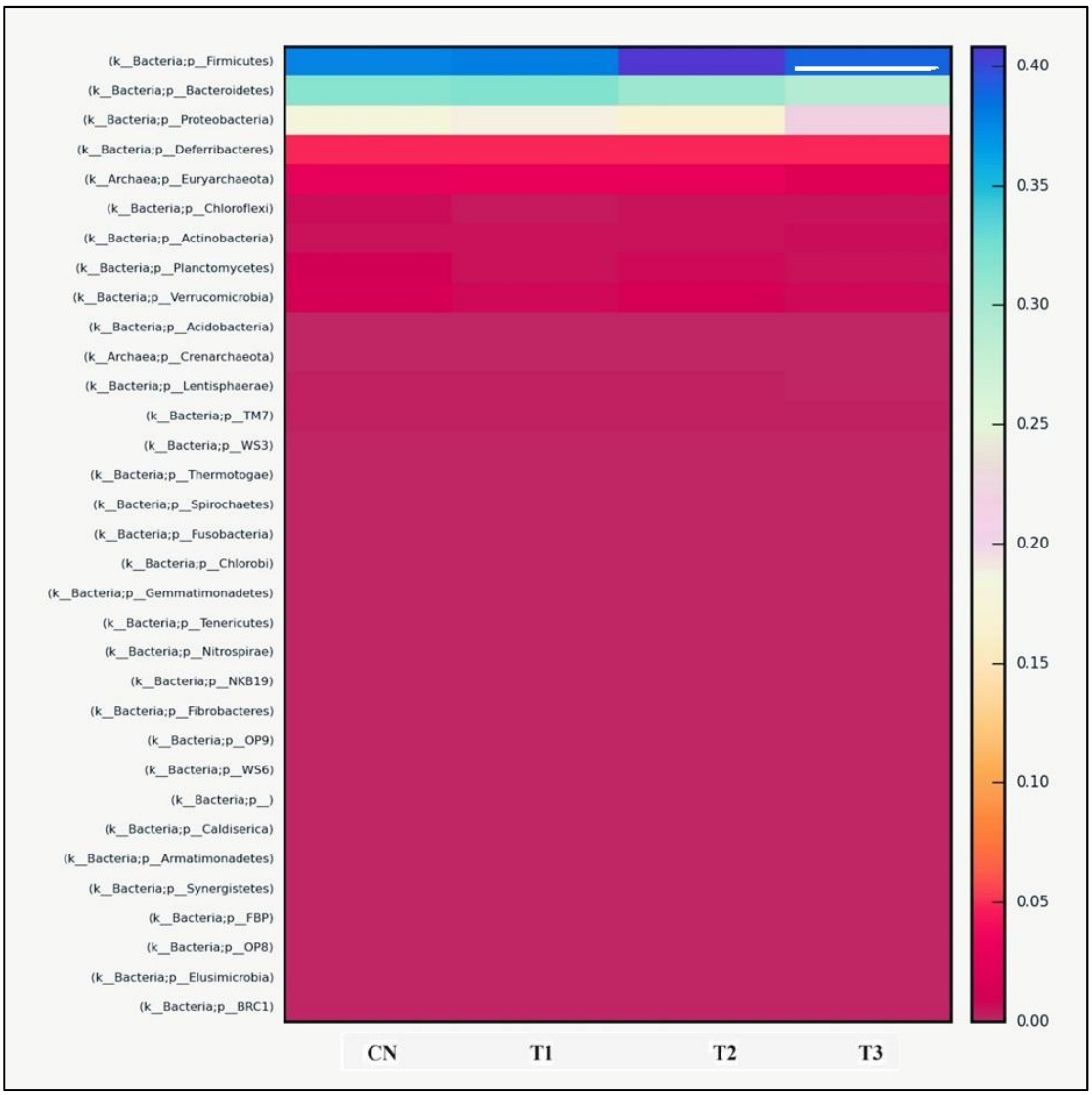


The X- axis is the number of sequences per sample and the Y – axis is the diversity index. CN: Control, T1: Thermal stress, T2: Vitamin C supplemented, T3: Vit C+ Thermal stress

4.1.14. Heatmap showing the relative abundance of phyla

The heatmap analysis of silkworm gut bacteria showed the specific and shared bacterial taxa. Bacteroidetes, Firmicutes and Proteobacteria were identified as the dominant phyla presented in all four groups.

Figure 4. 13: The representation of phyla in the larval gut of *B. mori* in both control and treated groups is depicted in the relative abundance chart. Each column corresponds to a gut sample, and each row represents a microbial taxon, with the shading indicating the relative abundance (Red = Lower abundance and Blue = Higher abundance).



4.1.15. Phylogenetic Analysis

Krona charts were used to summarise the total quantitative phylogenetic data for the gut tissue symbionts under control and treated groups. This interactive graphic illustrated the six-level taxonomic groupings and explained the bacterial diversity of each category.

Figure 4.14: Krona chart representation of control *B. mori* bacterial taxa (CN).

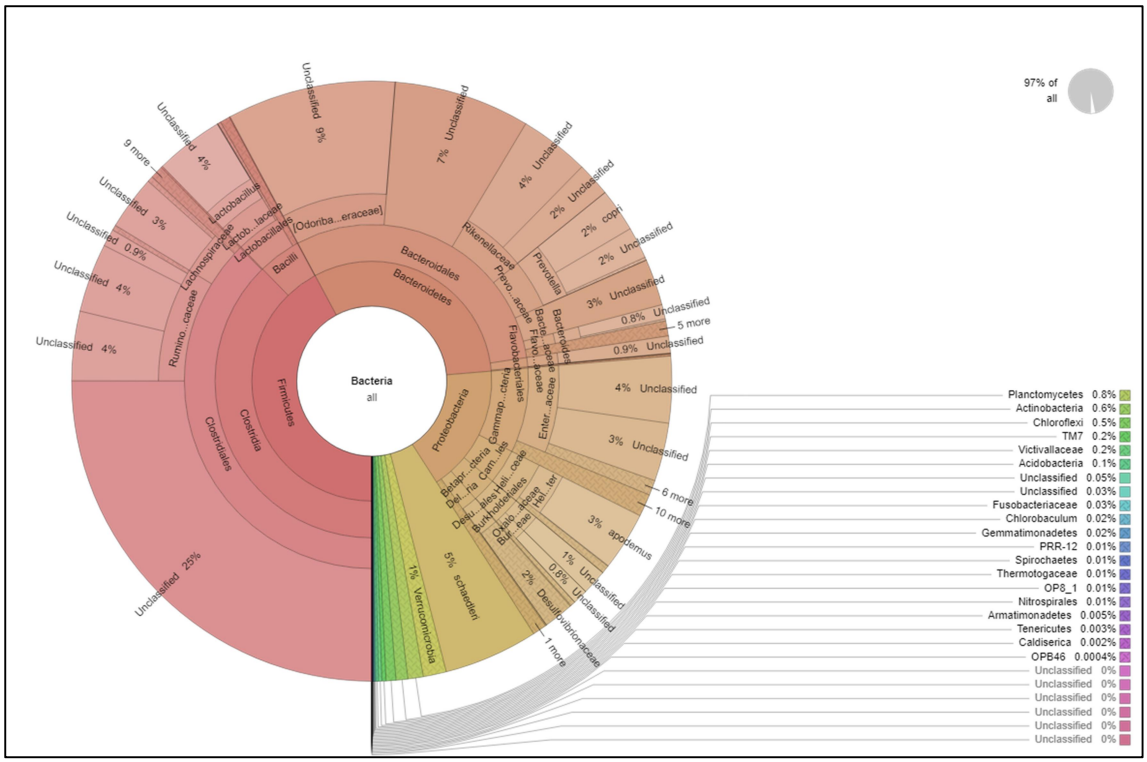


Figure 4.15: Krona chart representation of thermal stress exposed *B. mori* bacterial taxa (T1).

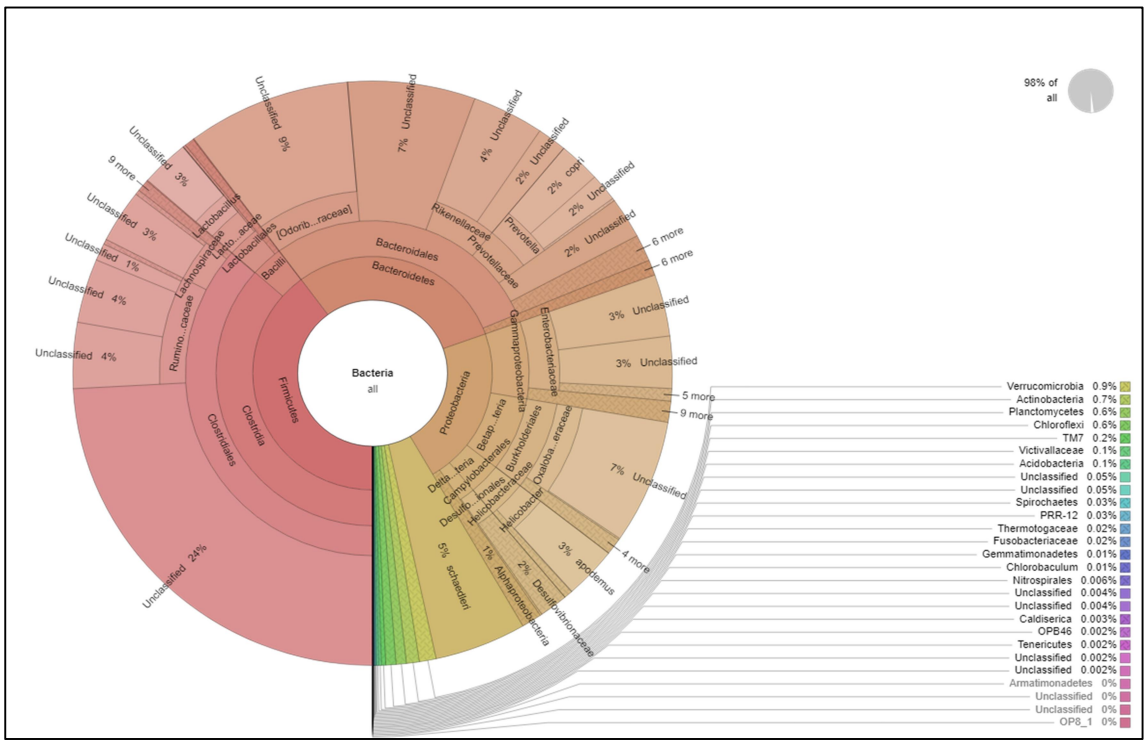


Figure 4.16: Krona chart representation of Vitamin C supplemented *B. mori* bacterial taxa (T2).

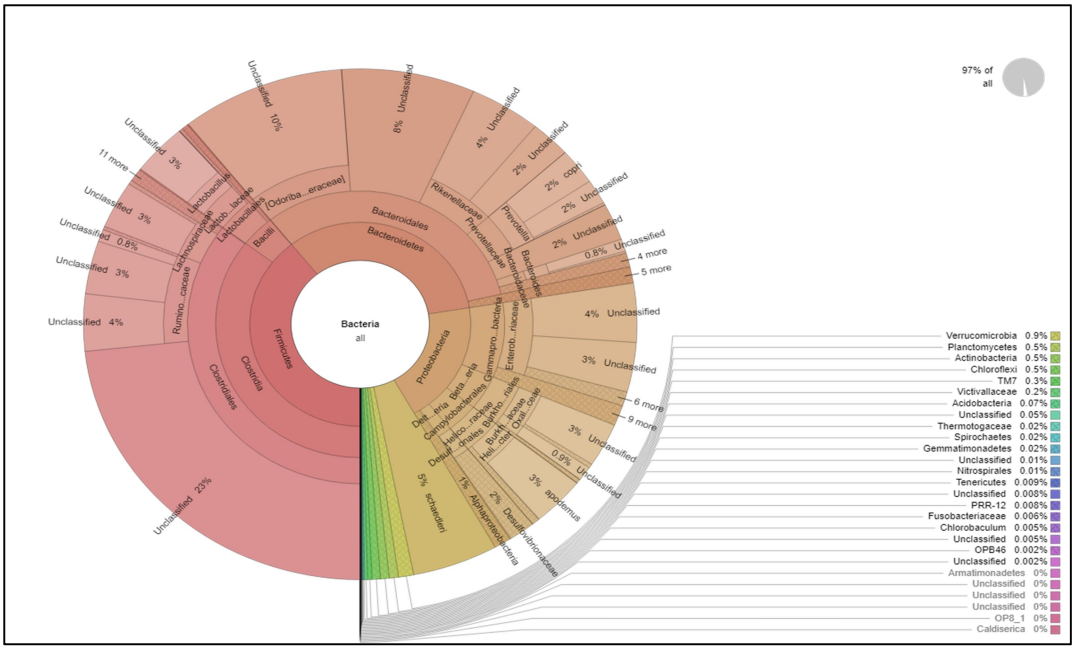


Figure 4.17: Krona chart representation of Vitamin C + thermal stress exposed *B. mori* bacterial taxa (T3).

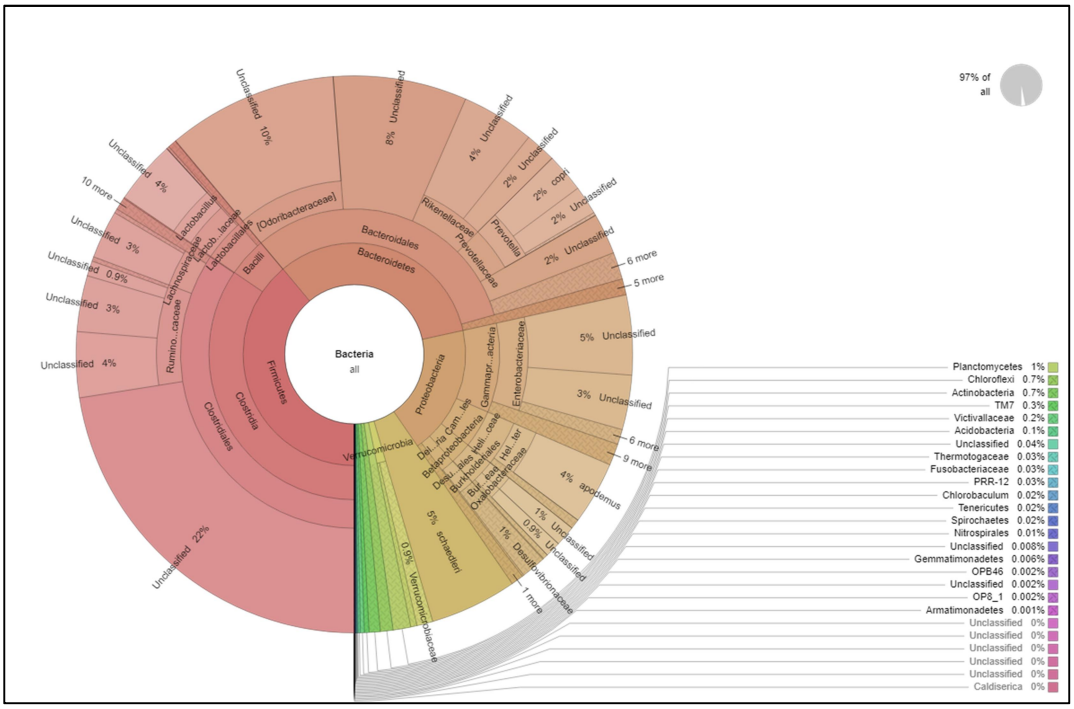


Figure 4.16: Krona chart representation of Vitamin C supplemented *B. mori* bacterial taxa (T2).

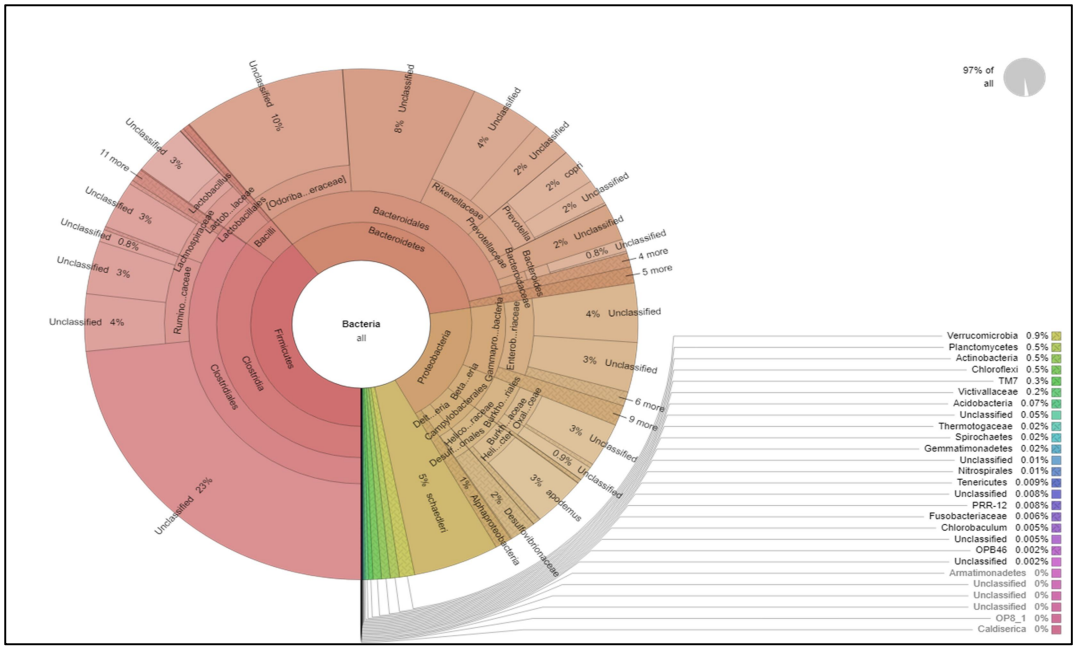
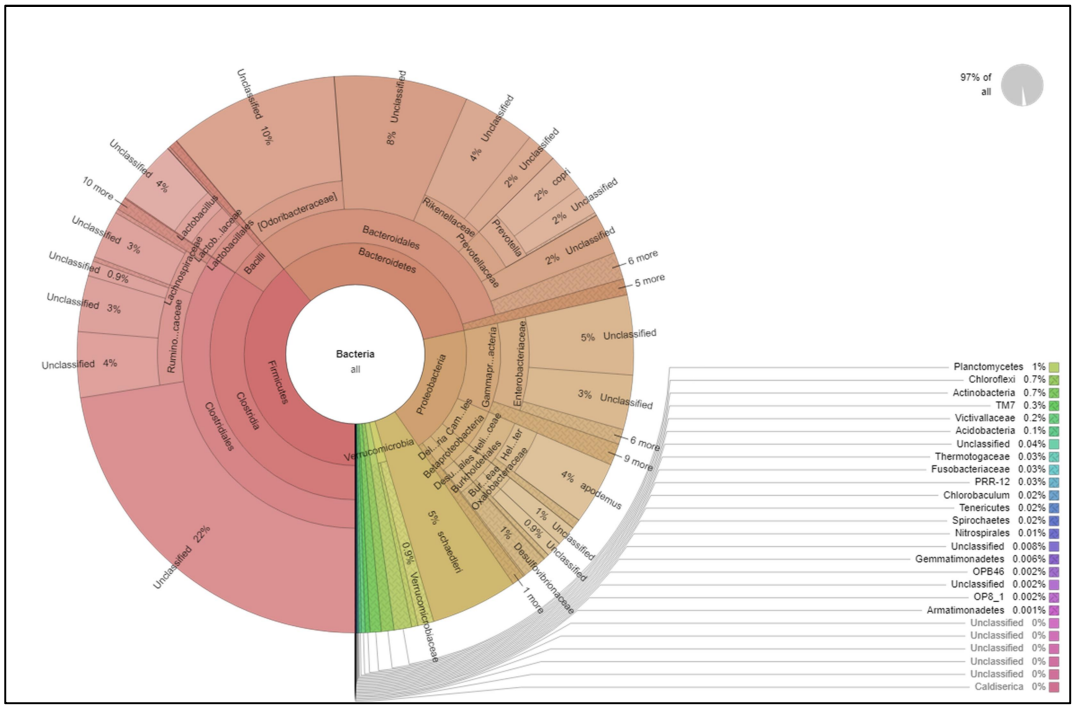


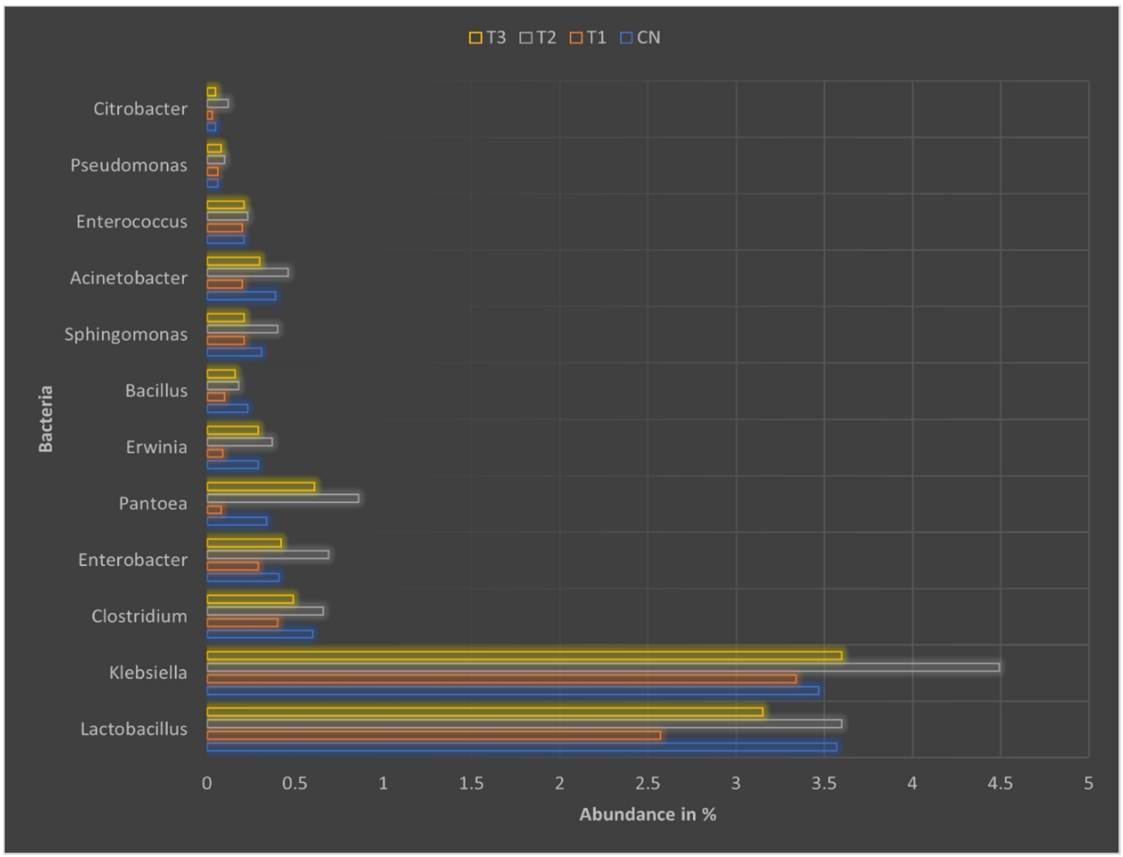
Figure 4.17: Krona chart representation of Vitamin C + thermal stress exposed *B. mori* bacterial taxa (T3).



4.1.16. Abundance of beneficial bacteria present in the gut of *B. mori* of control and treated groups.

Lactobacillus, Klebsiella, Clostridium, Enterobacter, Pantoe, Erwinia, Sphingomonas, Acinetobacter, Enterococous, Pseudomonas and Citrobacter are some of the beneficial bacteria present in the gut of silkworm for their growth and development. These bacteria can produce some digestive enzymes, minerals and nutrients which helps the larvae for absorption and metabolism. From analysing the abundance of composition of these bacteria from all the four groups it was found that all those bacterial compositions were declined in the thermal stress exposed group (T1) and found an elevation in the composition of bacteria in the vitamin C supplemented group (T2). The abundance of bacterial composition in the group T3 (Vitamin C + Thermal stress) also showed an increase than that of group T2.

Figure 4.18: Comparative analysis of abundance of useful bacteria presents in control and treated groups (CN, T1, T2 and T3)



CN-Control, T1-Thermal stress, T2- Vit C Supplemented, T3- Thermal stress + Vit C Supplemented.

4.2. MORPHOMETRIC PARAMETERS OF LARVAE

4.2.1. Total body length of silkworm larvae

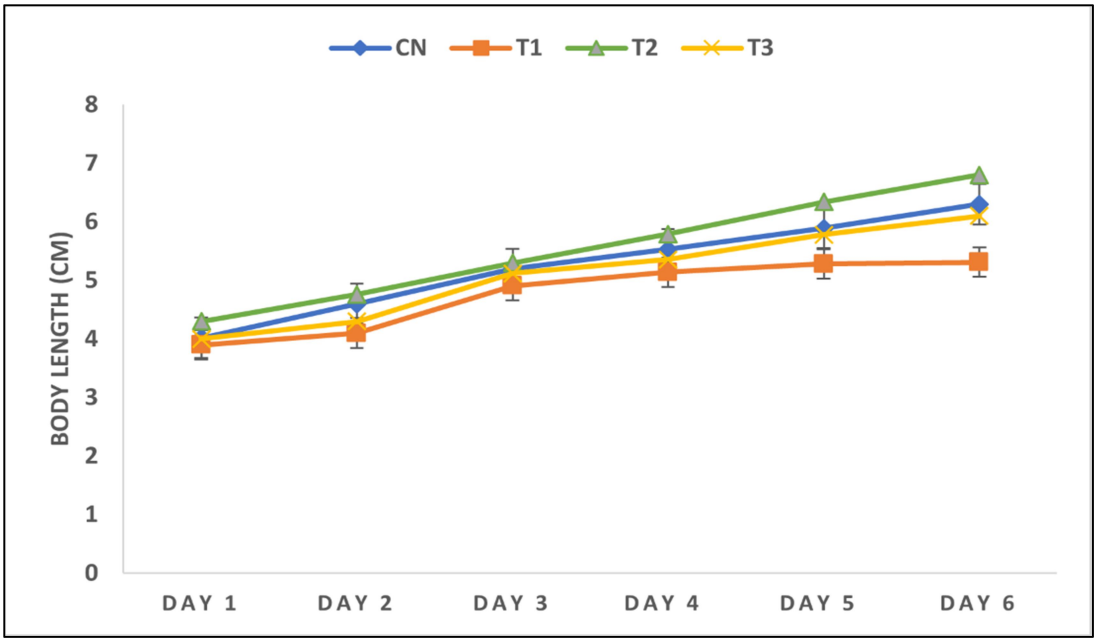
Throughout the experimental duration, the total body length of fifth instar larvae was regularly assessed. The findings revealed a notable decrease in the body length of larvae within the thermal stress group, and a significant increase was observed in the group supplemented with vitamin C, as compared to the control. The average length of larvae was highest in the ascorbic acid supplemented group [T2] (5.54 ± 0.22^{a}) and was significantly reduced in the thermal stress exposed group [T1] (4.77 ± 0.2^{b}) than the other groups (Fig 4.2). The larval length of silkworm supplemented with ascorbic acid under thermal stress group [T3] (5.11 ± 0.22^{ab}) showed a significant recovery when compared to thermal stress exposed group. The table 4.10 displays the periodic length measurements of the fifth instar larvae of silkworm from Day 1 to Day 6 in both the control and treated groups.

Table 4.10: The average length of fifth instar silkworm larvae of control and experimental groups (in cm)

Days	CN	T1	T2	T3
	Control	Thermal stress	Vit C Supplemented	Thermal stress+ Vit C supplemented
Day 1	4.02±0.2	3.9±0.15	4.3±0.18	4±0.17
Day 2	4.6±0.13	4.1±0.13	4.76±0.19	4.3±0.31
Day 3	5.2±0.32	4.91±0.23	5.3±0.23	5.12±0.17
Day 4	5.53±0.24	5.14±0.16	5.79±0.21	5.36±0.25
Day 5	5.89±0.15	5.28±0.32	6.34±0.26	5.78±0.23
Day 6	6.3±0.16	5.31±0.26	6.8±0.27	6.1±0.21
Average length of silkworm larvae	5.25 ± 0.2^{ab}	4.77 ± 0.2^{b}	5.54 ± 0.22^{a}	5.11 ± 0.22^{ab}

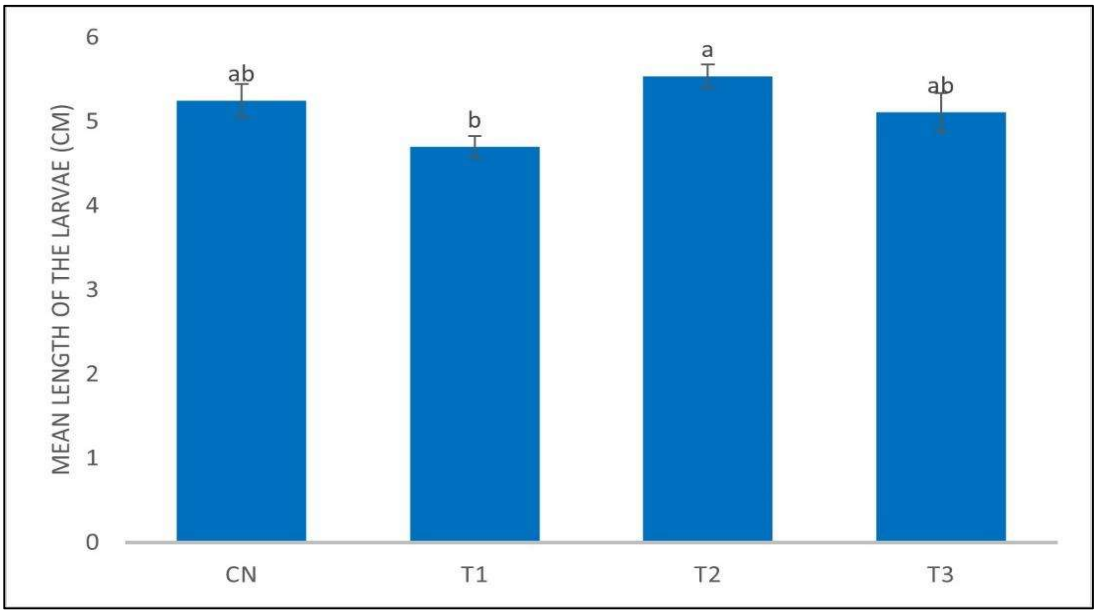
The variation between the groups was calculated by ANOVA followed by Tukey's test and significant at $P < 0.05$, n=6.

Figure 4.19: Periodic body length of fifth instar silkworm larvae from day 1 to day 6 in control and treated groups.



CN: Control, T1: Thermal stress, T2: Vitamin C supplemented, T3: Vit C+ Thermal stress

Figure 4.20: The average length of fifth instar silkworm larvae of control and treated groups.



CN: Control, T1: Thermal stress, T2: Vitamin C supplemented, T3: Vit C+ Thermal stress

4.2.2. Total weight of silkworm larvae

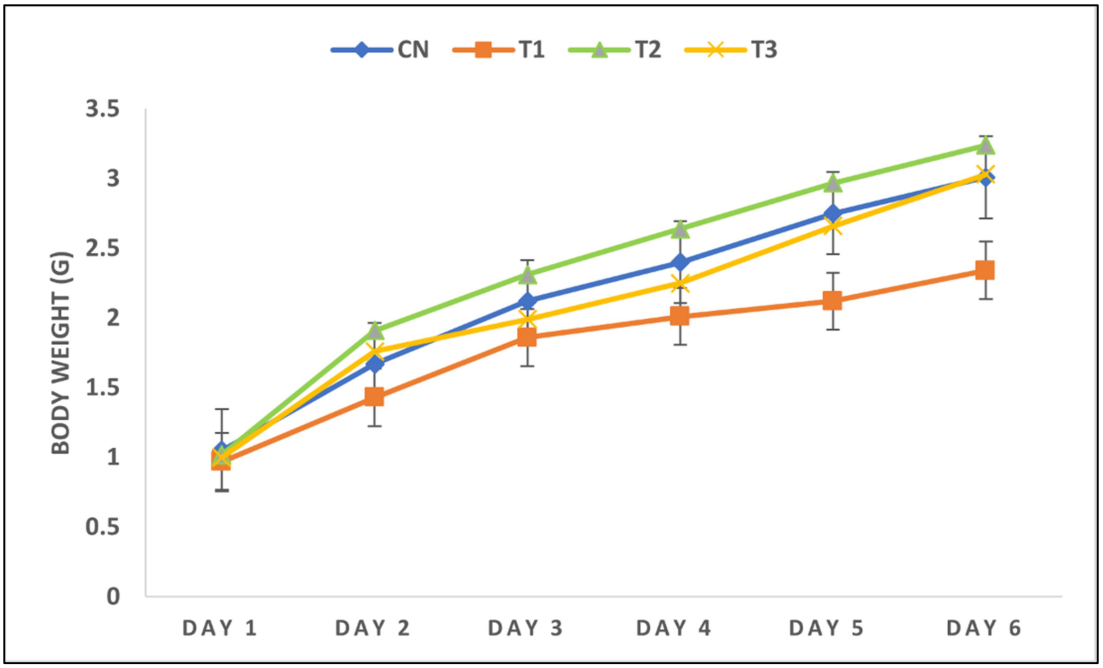
The body weight of fifth instar silkworm larvae were periodically measured and it was found that the body weight was significantly reduced in the group T1 (1.78 ± 0.19^{b}) than that of control larvae (2.16 ± 0.19^{ab}). A substantial increase in body weight was evident in group T2 (2.34±0.2a), and a significant rise was observed in group T3 (2.11±0.2ab). The table 4.2 presents the periodic weight measurements of the fifth instar larvae of silkworm from Day 1 to Day 6 in both the control and treated groups.

Table 4.11: The average weight of fifth instar silkworm larvae of control and experimental groups (in gm).

Days	CN	T1	T2	T3
	Control	Thermal stress	Vitamin C Supplemented	Thermal stress +VitaminC supplemented
Day 1	1.05±0.12	0.97±0.28	1.02±0.21	1±0.13
Day 2	1.67±0.21	1.43±0.21	1.91±0.26	1.76±0.15
Day 3	2.12±0.32	1.86±0.18	2.31±0.13	1.99±0.24
Day 4	2.4±0.14	2.01±0.13	2.64±0.17	2.25±0.21
Day 5	2.75±0.17	2.12±0.15	2.97±0.17	2.66±0.31
Day 6	3.01±0.18	2.34±0.21	3.24±0.3	3.03±0.18
Average weight of silkworm larvae	2.16 ± 0.19^{ab}	1.78 ± 0.19^{b}	2.34 ± 0.2^{a}	2.11 ± 0.2^{ab}

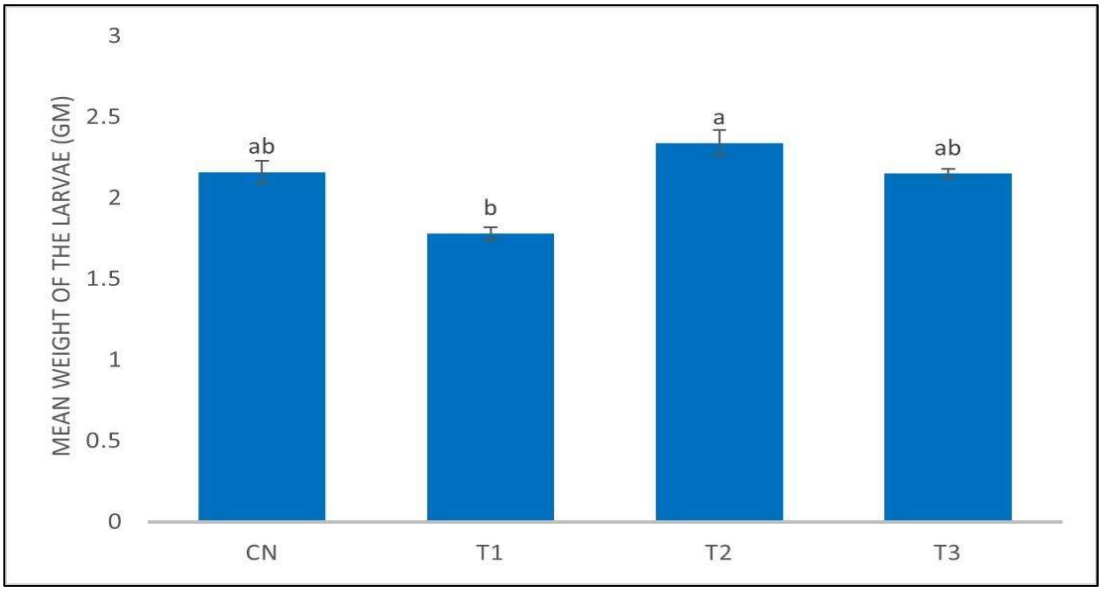
The variation between the groups was calculated by ANOVA followed by Tukey's test. a, b, c and d are the values differs significantly at $P<0.05$, n=6.

Figure 4.21: Periodic body weight of fifth instar silkworm larvae from day 1 to day 6 in control and treated groups.



CN: Control, T1: Thermal stress, T2: Vitamin C supplemented, T3: Vit C+ Thermal stress

Figure 4.22: The average weight of fifth instar silkworm larvae of control and treated groups.



CN: Control, T1: Thermal stress, T2: Vitamin C supplemented, T3: Vit C+ Thermal stress

4.3. BIOCHEMICAL ANALYSIS

The effect of supplementation of ascorbic acid on the silkworm physiology corresponding to the important digestive enzymes Amylase, protease, lipase and cellulase, total soluble protein content in the gut of silkworm larvae and morphometric characteristics of silkworm cocoon were recorded as follows.

Table 4. 12: Total protein and activity of digestive enzymes of control and experimental groups.

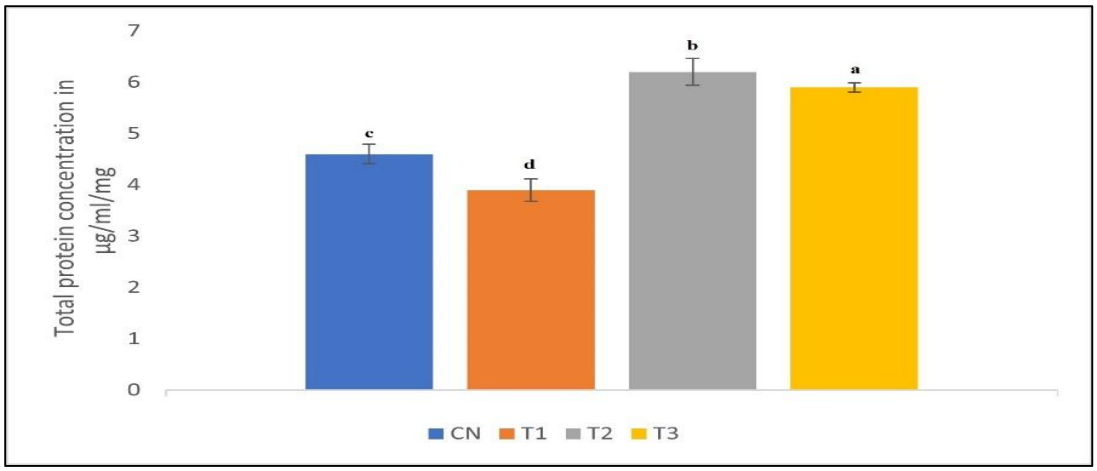
Groups	Protein	Amylase	Cellulase	Lipase	Protease
CN	4.6 ± 0.19^{c}	6.3 ± 0.13^{c}	3.49 ± 0.1^{c}	15.94 ± 0.22^{b}	16.15 ± 0.24^{b}
T1	3.9 ± 0.22^{d}	4.7 ± 0.06^{d}	2.44 ± 0.09^{d}	4.56 ± 0.15^{d}	13.3 ± 0.4^{c}
T2	6.2 ± 0.26^{b}	8.9 ± 0.035^{a}	4.88 ± 0.05^{b}	18.85 ± 0.51^{a}	17.95 ± 0.32^{a}
T3	5.9 ± 0.09^{a}	8.23 ± 0.17^{b}	4.19 ± 0.19^{b}	12.59 ± 0.43^{c}	14.57 ± 0.08^{c}

The variation between the groups was calculated by ANOVA followed by Tukey's test. a, b, c and d are the values differs significantly at $P<0.05$, n=6.

4.3.1. Activity of Protein

The total protein level was found to vary between 4.6 and 5.9 ug/ml/mg tissue. Protein content observed in groups T2 and T3 were significantly higher when compared to the Control group, however it considerably decreased in the temperature-exposed group (T1) compared to other groups. The results of the one-way ANOVA indicated that the differences among all experimental groups were deemed statistically significant, with p-values less than 0.001 and 0.05.

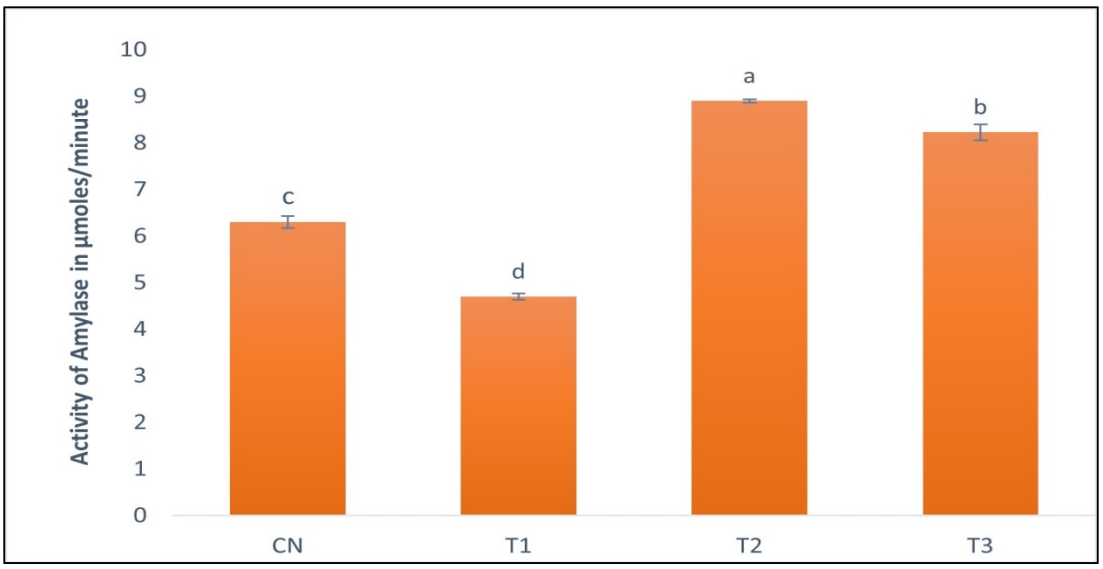
Figure 4.23: Total protein content of silkworm gut



4.3.2. Activity of Amylase

The amylase activity in the gut of silkworm of control (CN), heatshock (T1), ascorbic acid supplemented (T2) and heat shock along with ascorbic acid supplemented (T3) were 6.3±0.32µg/ml, 4.7±0.16µg/ml, 8.9±0.08µg/ml and 8.23±0.42µg/ml respectively. The amylase activity in the gut of silkworms exposed to thermal stress [T1] demonstrated lower activity compared to the other groups, including the untreated control batch. Ascorbic acid supplemented group [T2] showed a significant increase in the amylase activity than other groups. A significant recovery was noticed in Group T3 when compared to group T1.

Figure 4. 24: Activity of Amylase enzyme in the gut of B. mori

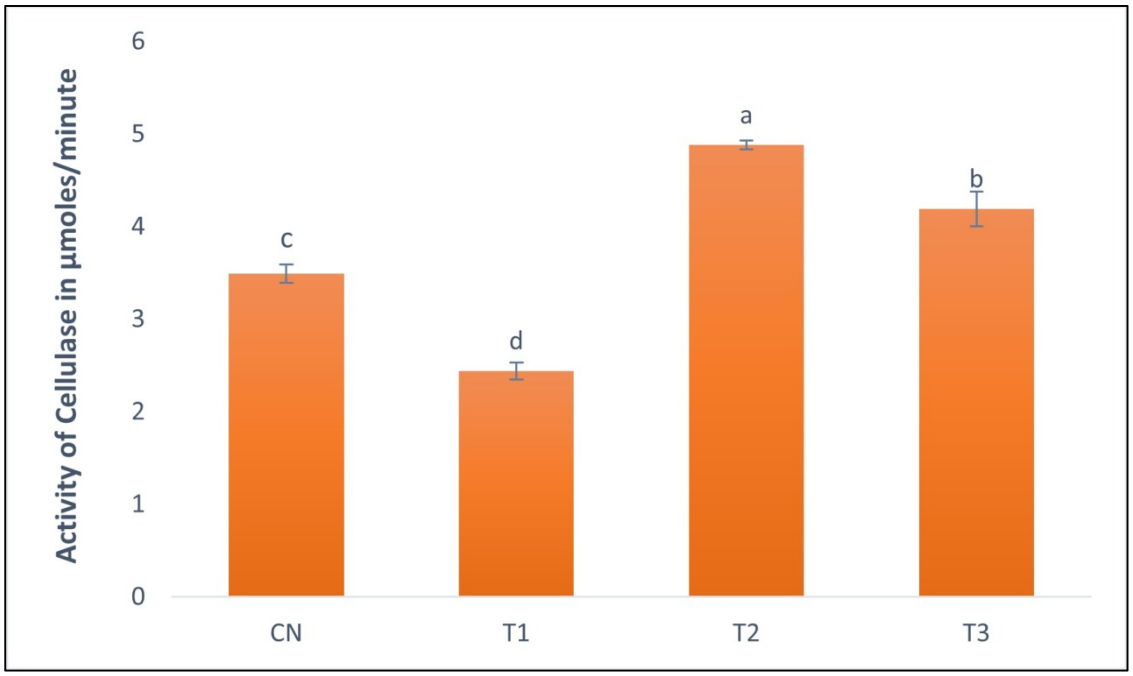


Total amylase activity in the gut of 5th instar larvae of Silkworm *Bombyx mori* (L.) Data are expressed as mean ± SEM of six observations. CN: Control., T1: Thermal stress exposed group., T2: Vitamin C supplemented., T3: Thermal Stress+ Vitamin C supplemented group.

4.3.3. Activity of Cellulase

Cellulase activity in the gut of silkworms was observed as 3.49±0.32µg/ml in the control group, 2.44±0.16µg/ml in the thermal stress exposed group, 4.88±0.08µg/ml in the group that received ascorbic acid supplements, and 4.19±0.42µg/ml in the thermal stress group that also received ascorbic acid supplements. Cellulase activity in the stomach of heat-shocked silkworms [T1] was lower than that of all groups, including untreated control batches. The cellulase activity was notably higher in the ascorbic acid-supplemented group [T2] compared to the other groups. When compared to Group T1, Group T3 showed a noticeable recovery.

Figure 4. 25: Activity of Cellulase enzyme in the gut of *B. mori*

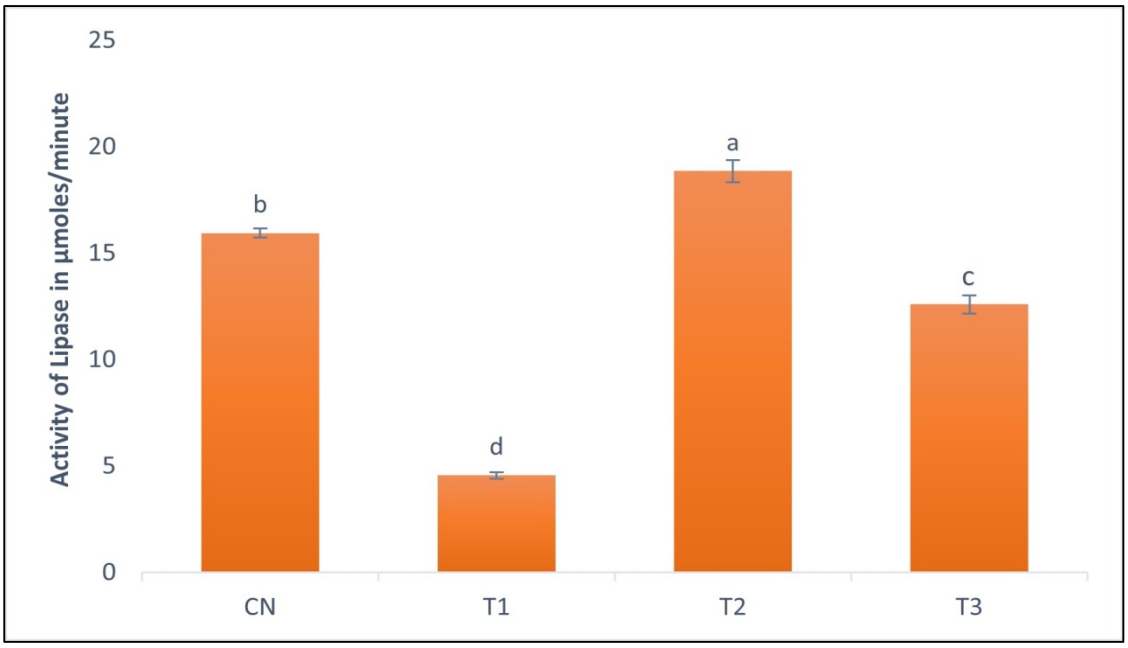


Total cellulase activity in the gut of 5th instar larvae of Silkworm *Bombyx mori* (L.) Data are expressed as mean ± SEM of six observations. CN: Control., T1: Thermal stress exposed group., T2: Vitamin C supplemented., T3: Thermal Stress+ Vitamin C supplemented group.

4.3.4. Activity of Lipase

The levels of lipase activity in the gut of silkworm were 15.94±0.55µg/ml (CN), 4.56±0.36µg/ml (T1), 18.85±0.9µg/ml (T2), and 12.59±0.97µg/ml (T3). In comparison to other groups, the lipase activity in the gut of heat-shocked silkworms [T1] was significantly decreased . The lipase activity was much higher in the group [T2] that was supplemented with ascorbic acid than in the other groups. Comparing Group T3 to Group T1, there was a discernible improvement.

Figure 4. 26: Activity of lipase enzyme in the gut of B. mori

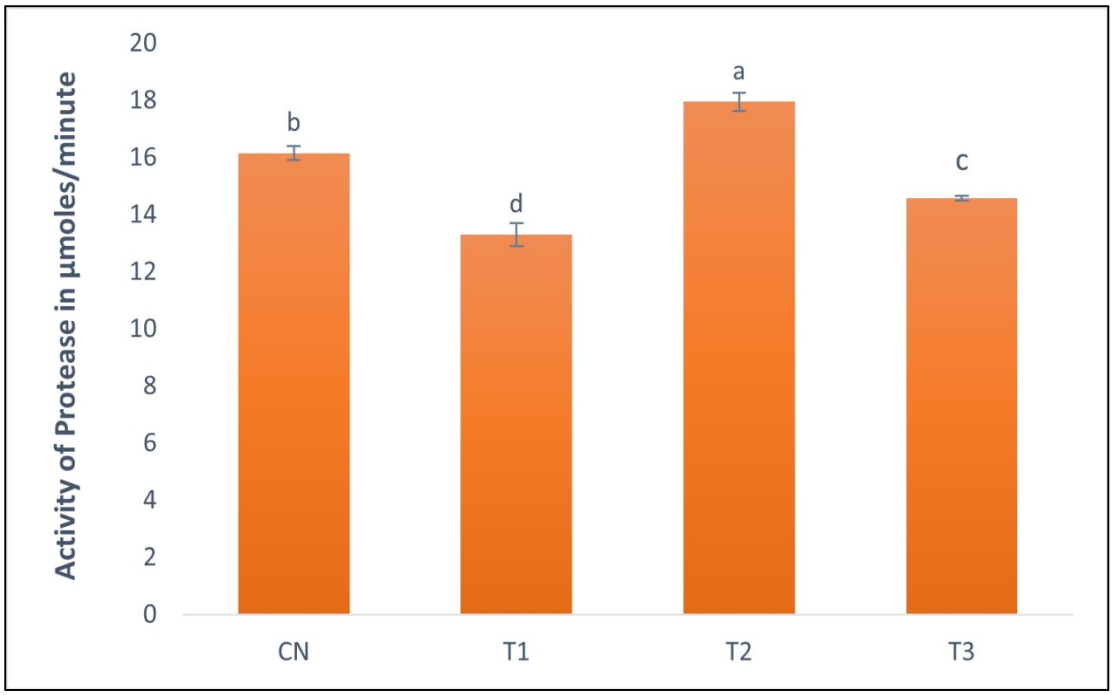


Total lipase activity in the gut of 5th instar larvae of Silkworm *Bombyx mori* (L.) Data are expressed as mean ± SEM of six observations. CN: Control., T1: Thermal stress exposed group., T2: Vitamin C supplemented., T3: Thermal Stress+ Vitamin C supplemenyted group.

4.3.5. Activity of Protease

The activity of protease enzyme was 16.15±0.54µg/ml (CN), 13.30±0.92µg/ml (T1), 17.95±0.72µg/ml (T2), and 14.57±0.18µg/ml (T3) in the gut of silkworm. Larvae subjected to thermal shock (T1) had less lipase activity in their guts than other groups, including control batches. The protease activity was markedly higher in the ascorbic acid-supplemented group (T2) compared to the other groups. Comparing T3 group to T1, a considerable recovery was seen.

Figure 4. 27: Activity of protease enzyme in the gut of *B. mori*



Total protease activity in the gut of 5th instar larvae of Silkworm *Bombyx mori* (L.) Data are expressed as mean ± SEM of six observations. CN: Control., T1: Thermal stress exposed group., T2: Vitamin C supplemented., T3: Thermal Stress+ Vitamin C supplemenyted group.

4.4. MOLECULAR ANALYSIS

The relative mRNA expression levels of Hsp 20.4, Hsp 40, Hsp 70 and Hsp 90 genes were quantified using real time qPCR of gut tissue of the fifth day of fifth instar larvae of control and experimental groups.

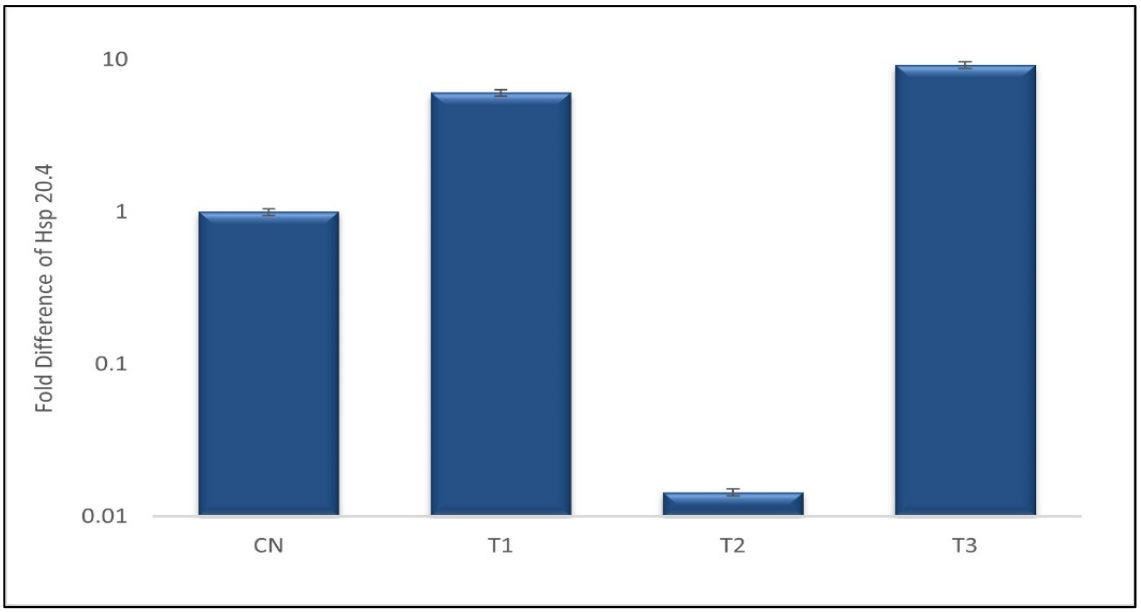
4.4.1. Expression of Hsp 20.4 gene in the larval gut

The findings from the study indicate notable differences in the expression of the Hsp 20.4 gene transcript among different groups of silkworm larvae. Specifically, the results reveal a significant upregulation of the Hsp 20.4 gene transcript in the gut of silkworm larvae belonging to groups T1 and T3 when compared to both the control group and the T2 group. This suggests that thermal stress contribute to a heightened expression of the Hsp 20.4 gene in the silkworm gut. Interestingly, the T2 group supplemented with vitamin C exhibited a comparatively lower expression of the Hsp 20.4 gene transcript in contrast to the other groups.

Table 4.13: Expression of Hsp 20.4 gene in the gut of silkworm larvae of Control and treated groups.

Groups	Control (CN)	Heat Shock (T1)	Vitamin C supplemented (T2)	Heat shock + Vitamin C Supplemented (T3)
Hsp 20.4	31.61	29.61	23.26	31.15
b- Actin	34.4	29.8	32.16	30.73
Normalised expression value	-2.79	-0.19	-8.9	0.42
ΔΔCt=ΔCt treated -ΔCt untreated	0	2.6	-6.11	3.21
Fold difference in VTL relative to untreated = 2-ΔΔCt	1	6.0606	0.0144	9.2535

Figure 4.28: Fold difference of Hsp 20.4 in the gut of silkworm larvae of Control and treated groups



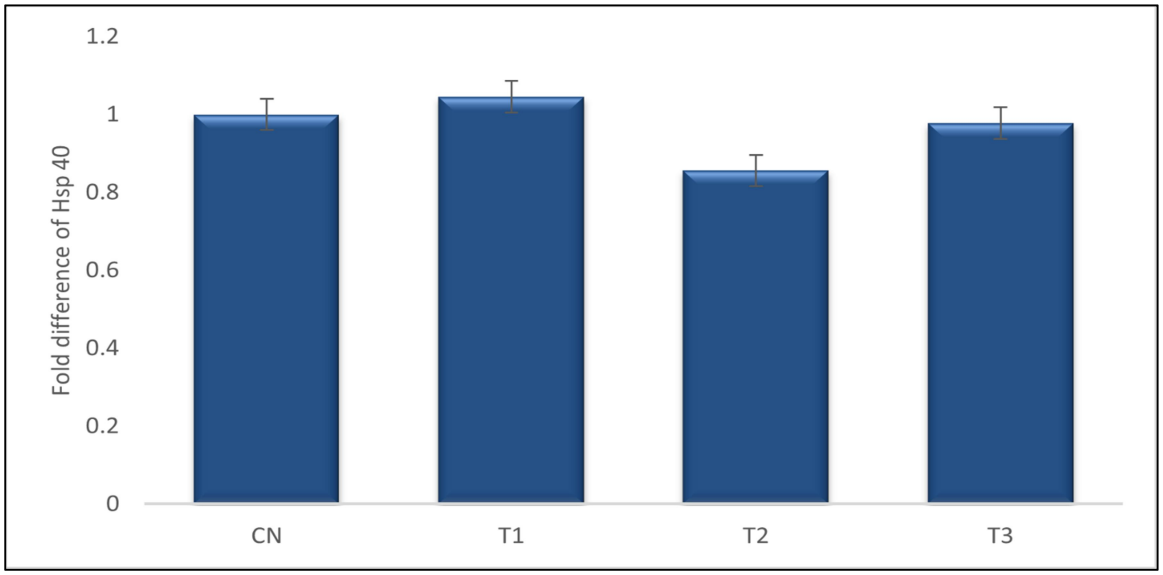
4.4.2. Expression of Hsp 40 gene in the larval gut

The results indicated that the supplementation of ascorbic acid (T2) to the silkworm larvae led to a downregulation of Hsp 40 gene expression compared to other groups, including the control. Conversely, the group exposed to heat exhibited (T1) an elevation in the expression of the Hsp 40 gene. This upregulation in response to heat exposure aligns with the well-established role of heat shock proteins, including Hsp 40, in cellular stress responses.

Table 4.14: Expression of Hsp 40 gene in the gut of silkworm larvae of Control and treated groups

Groups	**Control (CN)**	**Heat Shock (T1)**	**Vitamin C supplemented (T2)**	**Heat shock + Vitamin C Supplemented (T3)**
Hsp 40	35.07	31.16	27.53	30.04
b- Actin	34.4	29.8	32.16	30.73
Normalised expression value	0.67	1.36	-4.63	-0.69
Fold difference	1	1.0456	0.8560	0.9775

Figure 4.29: Fold difference of Hsp 40 in the gut of silkworm larvae of Control and treated groups



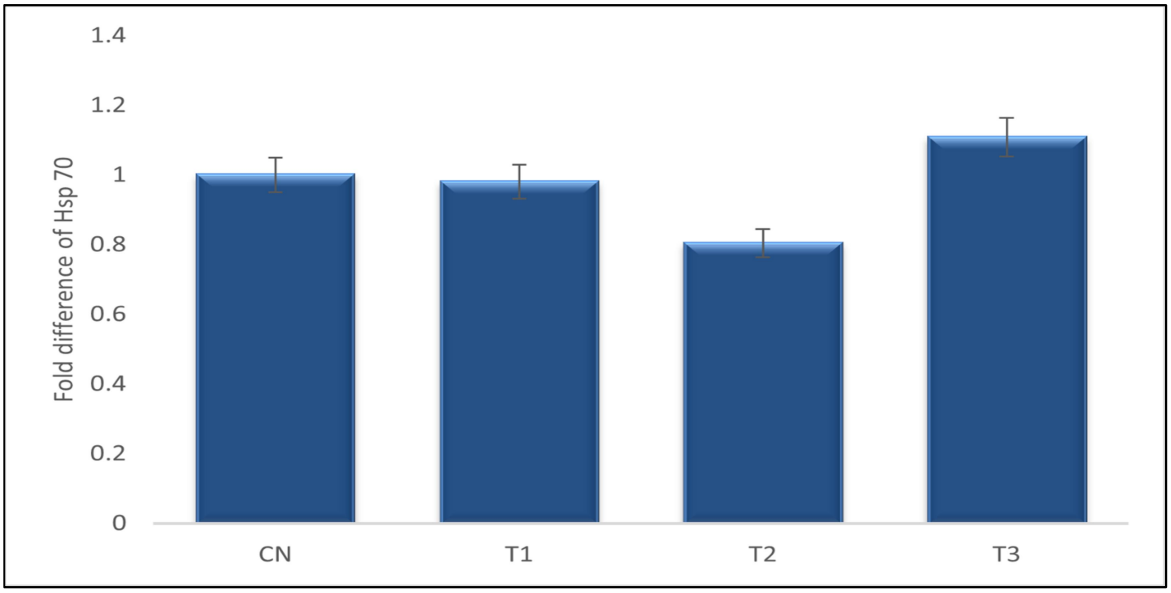
4.4.3. Expression of Hsp 70 gene in the larval gut

There was an increase in the Hsp 70 gene expression in the gut of silkworm larvae of group T1 compared to the control group and other groups. The T2 group exhibited a decrease in gene expression, while the T3 group demonstrated a significantly higher expression than the control group.

Table 4.15: Expression of Hsp 70 gene in the gut of silkworm larvae of Control and treated groups

Groups	**Control (CN)**	**Heat Shock (T1)**	**Vitamin C supplemented (T2)**	**Heat shock + Vitamin C Supplemented (T3)**
Hsp 70	30.12	29.23	25.84	38.12
b- Actin	30.73	29.8	32.16	34.4
Normalised expression value	-0.61	-0.57	-6.32	3.72
Fold difference	1	0.9808	0.8034	1.1081

Figure 4.30: Fold difference of Hsp 70 in the gut of silkworm larvae of Control and treated groups



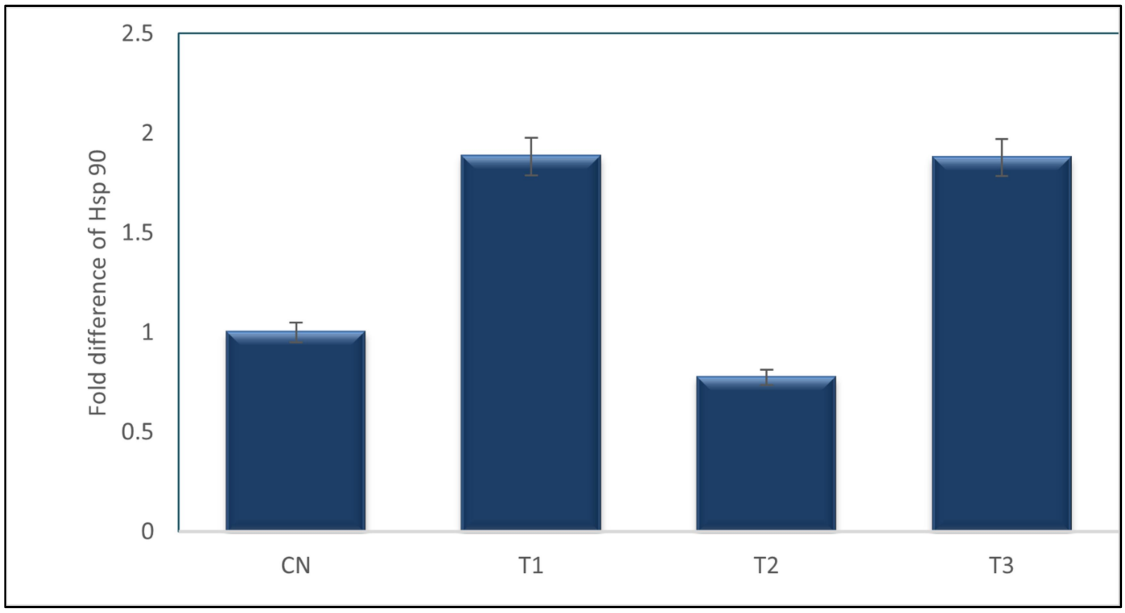
4.4.4. Expression of Hsp 90 gene in the larval gut

Within the larval gut, Group T1 exhibited an elevation in the expression of the Hsp 90 gene compared to both the control group and the other experimental groups. Conversely, Group T2 exhibited a decrease in gene expression, while Group T3 displayed a notably higher expression level than the control group.

Table 4.16: Expression of Hsp 90 gene in the gut of silkworm larvae of Control and treated groups

Groups	**Control (CN)**	**Heat Shock (T1)**	**Vitamin C supplemented (T2)**	**Heat shock + Vitamin C Supplemented (T3)**
Hsp 90	28	26.31	26.62	26.99
b- Actin	32.16	29.8	34.4	30.73
Normalised expression value	-4.16	-3.49	-7.78	-3.74
Fold difference	1	1.8828	0.7738	1.8782

Figure 4.31: Fold difference of Hsp 90 in the gut of silkworm larvae of Control and treated groups



4.5. HISTOLOGICAL ANALYSIS OF SILKWORM LARVAL GUT

The midgut constitutes the essential functional center of the alimentary canal, where food digestion and nutrient absorption take place. The HE staining cross section of gut of fifth instar silkworm larvae of control and treated groups were done to evaluate the structural and morphological changes of larval gut under the experimental conditions.

4.5.1. Gut histology of fifth instar larvae of control (CN)

The histological architecture of the gut of silkworm larvae of control showed the presence of intact epithelial cells (EC, orange arrows), goblet cells (G, green arrows) and stem cells (SC, black arrows). The basal lamina (BL, blue arrows) is continuously found underlying and supporting the epithelial cells.

Figure 4.32: Gut histology of fifth instar larvae of control (CN)

a. **b.**

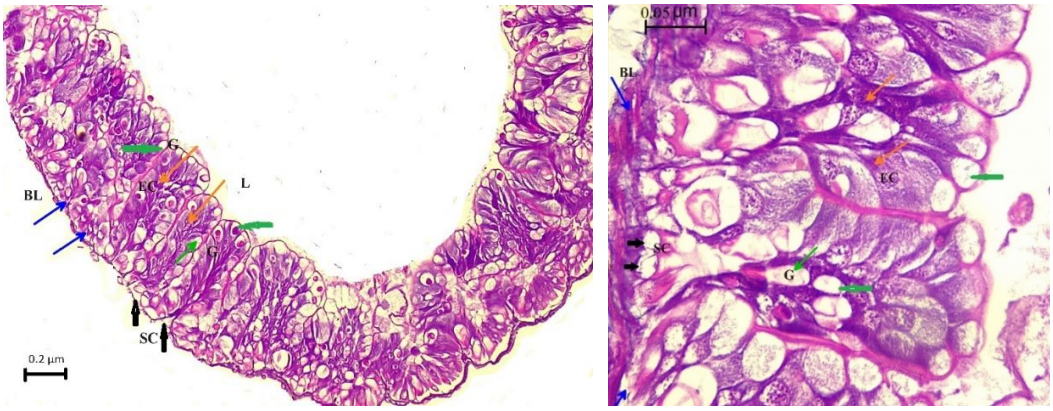


Fig 4.32: a. Low power (10x) photomicrograph of HE staining of section of control midgut epithelium of silkworm *B. mori*. **b.** High power (40x) photomicrograph of the control midgut epithelium of silkworm *B. mori.* L represents lumen of the gut, EC represents epithelial cells, G represents goblet cells, BC represents basal lamina and SC represents stem cells. Bar = 0.2µm.

- Basal Lamina
- Epithelial cell columnae
- Goblet cells
- Stem cells

4.5.2. Gut histology of fifth instar larvae of thermal stress group (T1)

The histomorphology of midgut of silkworm larvae exposed to high temperature showed the presence of various pathological changes. The interesting cellular change noticed was the fusion of the epithelial cells (orange arrows) and the widening of the goblet cells (green arrows), with accumulation of some secretory granules (* star). The basal lamina and stem cells were found to be intact.

Figure 4.33: Gut histology of fifth instar larvae of thermal stress group (T1)

a. **b.**

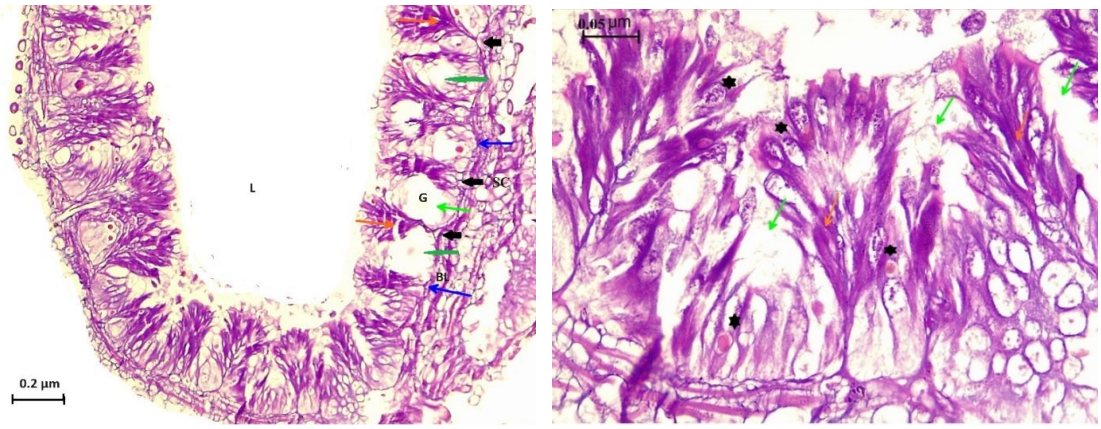


Fig 4.33: a. Low power (10x) photomicrograph of HE staining of section of temperature exposed midgut epithelium of silkworm larvae showing fusion of epithelial cells and widening of the goblet cell spanning the major area of epithelium. Sloughing off the epithelial cells at the apical end is also observed. **b.** High power (40x) photomicrograph of the temperature exposed midgut epithelium of silkworm *B. mori.* L represents lumen of the gut, EC represents epithelial cells, G represents goblet cells, BC represents basal lamina and SC represents stem cells. Bar = 0.2µm.

- Basal Lamina
- Epithelial cell columnae
- Goblet cells
- Stem cells

4.5.3. Gut histology of fifth instar larvae of vitamin C supplemented group (T2)

The histomorphology of vitamin C supplemented gut epithelium also showed the presence of intact epithelial cells, goblet cells and stem cells which was supported by the basal lamina.

Figure 4.34: Gut histology of fifth instar larvae of vitamin C supplemented group (T2)

a. **b.**

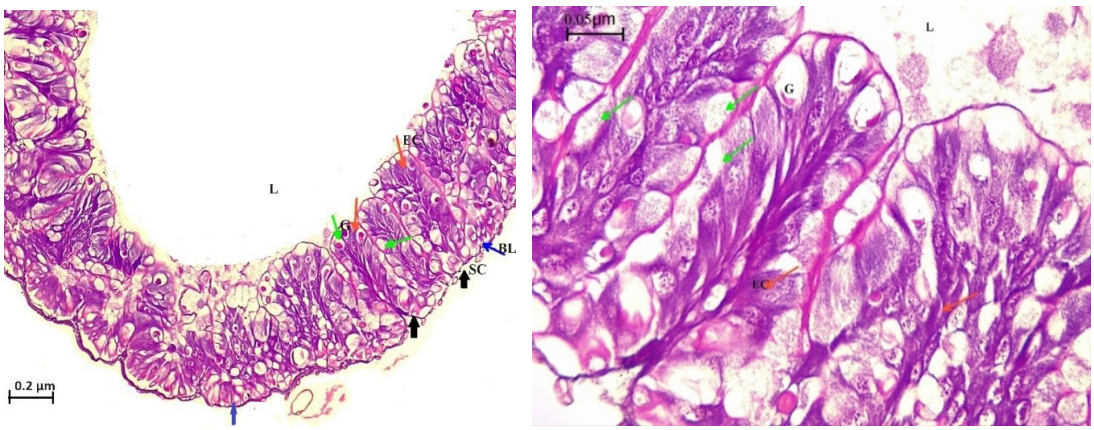


Fig 4.34: a. Low power (10x) photomicrograph of HE staining of section of vitamin C supplemented midgut epithelium of silkworm *B. mori*. **b**. High power (40x) photomicrograph of the vitamin C supplemented midgut epithelium of silkworm *B. mori.* L represents lumen of the gut, EC represents epithelial cells, G represents goblet cells, BC represents basal lamina and SC represents stem cells. Bar = 0.2µm.

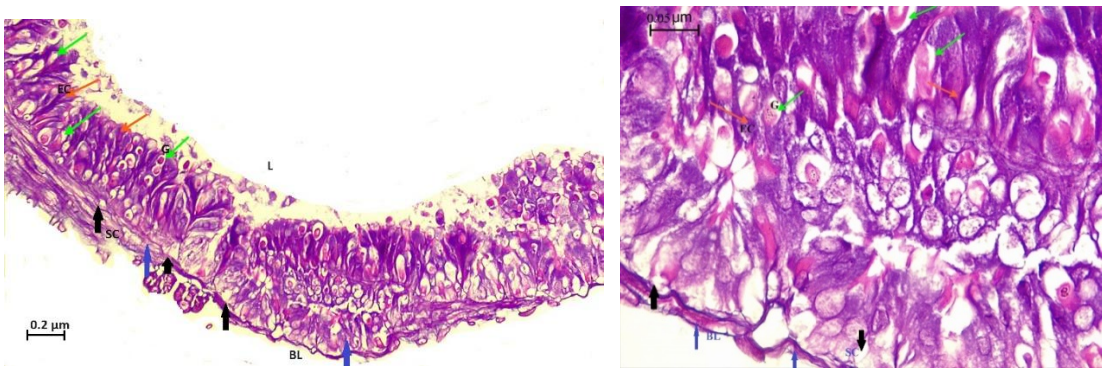
- Basal Lamina
- Epithelial cell columnae
- Goblet cells
- Stem cells

4.5.4. Gut histology of fifth instar larvae of Vitamin C + Thermal Stress group (T3)

The histomorphology of midgut of silkworm larvae of vitamin C supplemented under thermal stress showed a recovery sign in the gut epithelium with goblet cells retrieving back to its normal histomormorphological characters. A reorganisation of epithelial cells to its normal architecture was observed. The goblet cells got reduced in its width (green arrows) and was spaced out between epithelial cells, although it was found to be highly secretory.

Figure 4.35: Gut histology of fifth instar larvae of Vitamin C + Thermal Stress group (T3)

a. **b.**



4.35: a. Low power (10x) photomicrograph of HE staining of section of vitamin C + thermal stress midgut epithelium of silkworm *B. mori.* Histoarchitecture showing an alleviation in the morphology of midgut epithelium. **b.** High power (40x) photomicrograph of the control midgut epithelium of silkworm *B. mori* showing a recovery in its architecture. L represents lumen of the gut, EC represents epithelial cells columnae, G represents goblet cells, BC represents basal lamina and SC represents stem cells. Bar = 0.2µm.

- Basal Lamina
- Epithelial cell columnae
- Goblet cells
- Stem cells

4.6. QUANTITY AND QUALITY EVALUATION OF COCOON

4.6.1. Morphometric parameters of cocoons

The cocoon length, width, weight, cocoon shell weight, percent of good cocoons and cocoon shell percentage were calculated for analysing the changes in the cocoon in the control and experimental group. It was found that the quantity of cocoon produced in the group T3 larvae (heat shock + ascorbic acid supplemented) was significantly increased than that of the group T1 (heat shock). The morphometric parameters of cocoons of group T2 were increased significantly and decreased significantly in the T1 group.

Table. 4.17: "Morphometric parameters of cocoons produced by silkworm larvae *Bombyx mori*"

Experimental groups	Control (CN)	Heat Shock (T1)	Ascorbic acid supplemented (T2)	Heat shock + Ascorbic acid Supplemented (T3)
Length of the cocoon	2.8 ± 0.19^{b}	2.3 ± 0.2^{c}	3.2 ± 0.09^{a}	2.9 ± 0.13^{b}
Width of the cocoon	1.3 ± 0.02^{b}	0.9 ± 0.07^{c}	1.6 ± 0.05^{a}	1.4 ± 0.04^{ab}
Average single Cocoon Weight (gm)	1.8 ± 0.31^{ab}	1.2 ± 0.17^{c}	2.2 ± 0.09^{a}	2.0 ± 0.39^{b}
Cocoon shell weight (gm)	0.8 ± 0.32^{c}	0.4 ± 0.26^{d}	1.1 ± 0.55^{a}	1.0 ± 0.42^{b}
Percent good cocoons	80%	63.3%	90%	83%
Cocoon shell percent	0.44	0.33	0.5	0.5

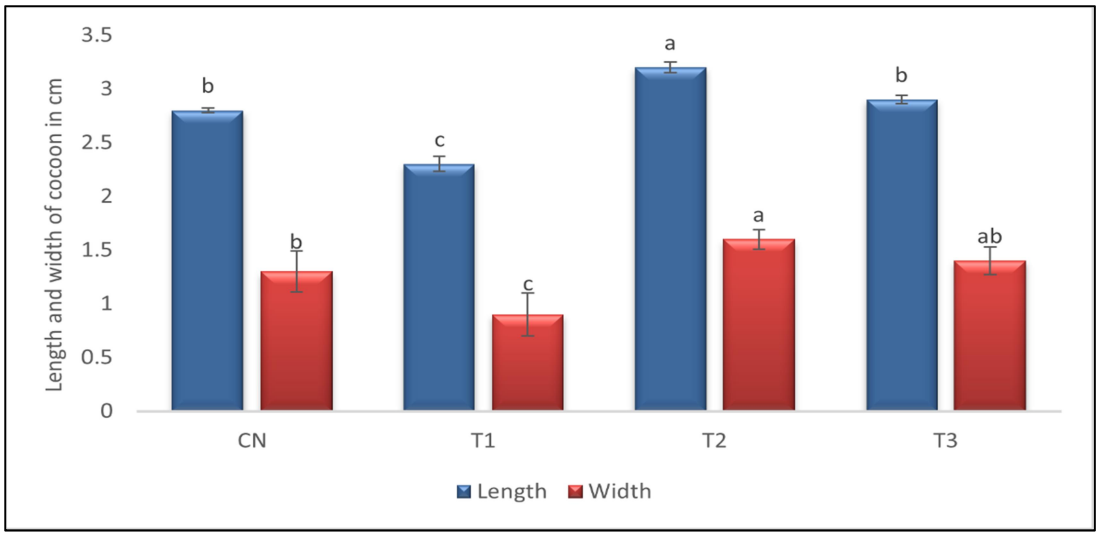
"The variation between the groups was calculated by ANOVA followed by Tukey's test. a, b, c and d are the values differs significantly at $P<0.05$, n=6".

4.6.1.1. Length and width of the Cocoon:

The average length in control was 2.8±0.19b, representing the baseline under standard conditions. Heat Shock (T1) showed a significant decrease to 2.3±0.2c, indicating a notable impact of heat shock on cocoon length. Ascorbic Acid Supplemented (T2) group demonstrated a significant increase to 3.2±0.09a, showcasing a positive effect of supplementation and Heat Shock + Ascorbic Acid Supplemented (T3) group showed a recovery to 2.9±0.13b, indicating a positive impact of ascorbic acid under heat shock conditions.

In the heat shock group (T1) cocoon width was reduced to 0.9±0.07c, highlighting the adverse effects of heat shock on cocoon dimensions compared to control (CN) 1.3±0.02b, and other groups. Ascorbic Acid supplemented (T2) cocoon width increased to 1.6±0.05a, showcasing the beneficial effect of supplementation. Moreover, the cocoon width of Heat Shock + Ascorbic Acid Supplemented (T3) group remained at 1.4±0.04ab, indicating a partial recovery compared to the heat shock group.

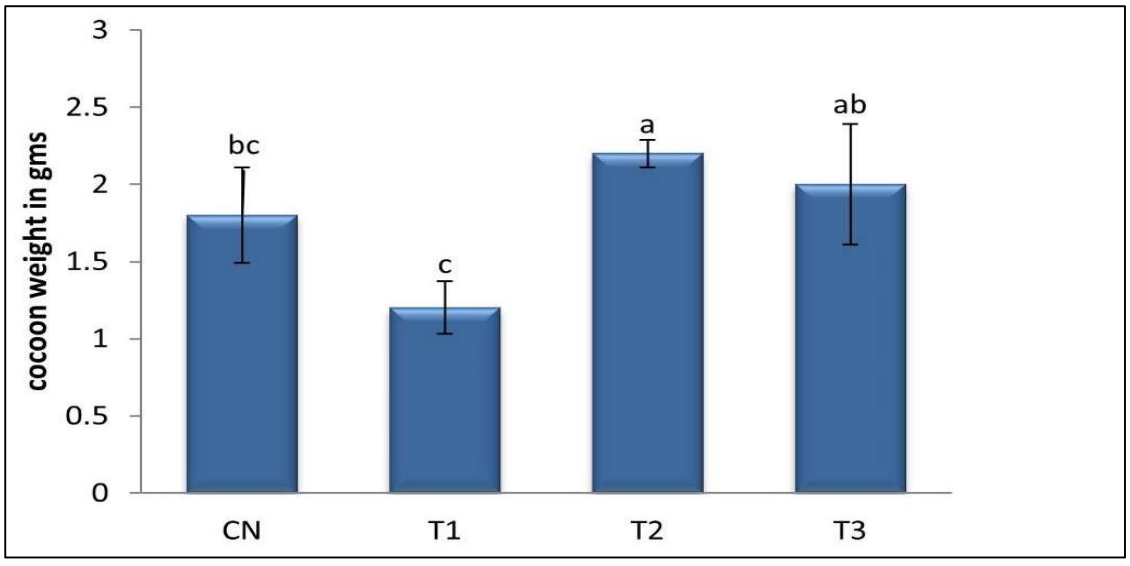
Figure 4. 36: Length and width of the cocoon



4.6.1.2. Average Single Cocoon Weight

The weight of average single cocoon was 1.8±0.31ab in control, reflecting the standard weight of individual cocoons in the control (CN). The average weight dropped to 1.2±0.17c, signifying a decline in cocoon weight under thermal stress group (T1). The average weight of Ascorbic Acid Supplemented (T2) group surged to 2.2±0.09a, showcasing an increase in cocoon weight with supplementation. Heat Shock + Ascorbic Acid Supplemented (T3) showed an increase in the weight to 2.0±0.39b, demonstrating a positive influence on cocoon weight even under thermal stress.

Figure 4.37: Average single cocoon weight



4.6.1.3. Cocoon Shell Weight

The cocoon shell weight was 0.8±0.32c in control, a characteristic measure for cocoons in normal conditions. Whereas, a substantial decrease to 0.4±0.26d was noted in Heat Shock (T1) group, indicating a considerable reduction in shell weight. Ascorbic Acid Supplemented (T2) showed an increase to 1.1±0.55a indicated a positive influence on cocoon shell weight with supplementation. An increase to 1.0±0.42b indicated a recovery in shell weight under combined heat shock and supplementation.

Figure 4.38: Cocoon shell weight

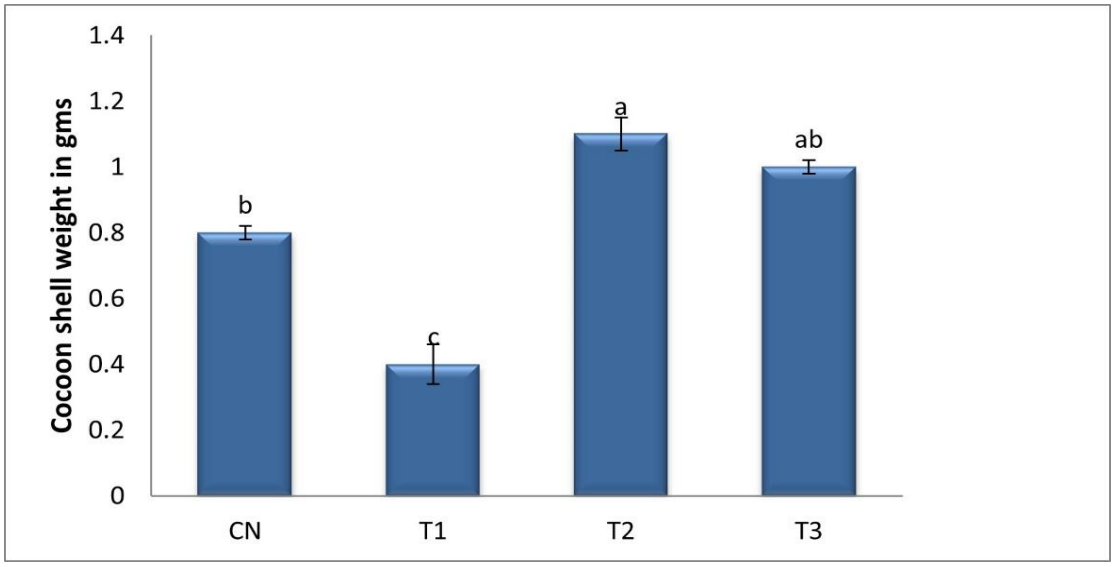
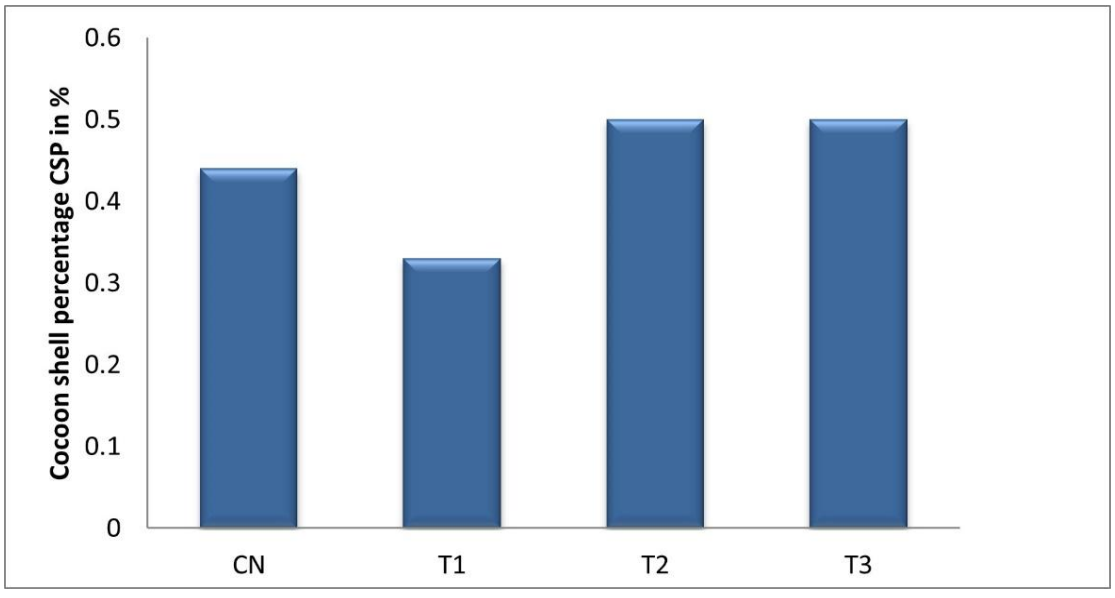


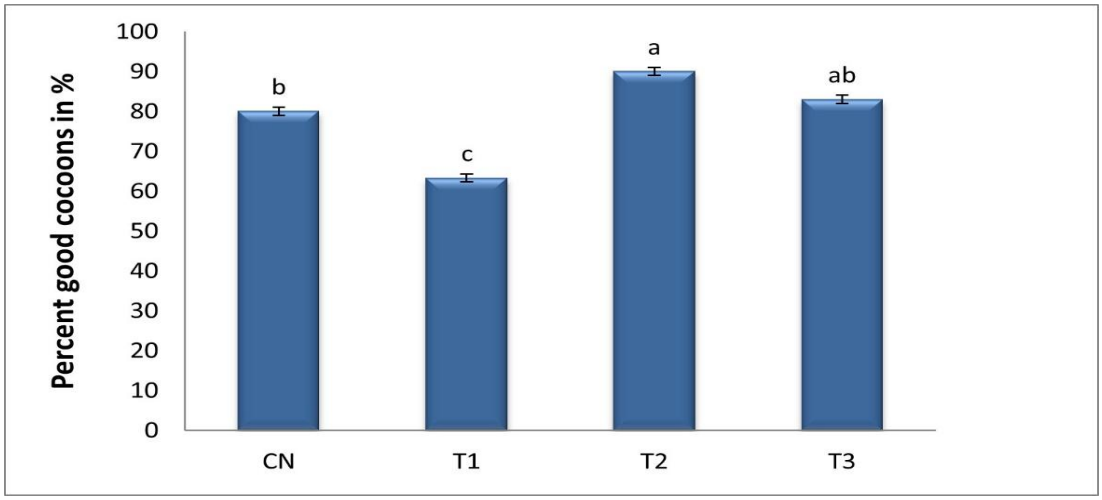
Figure 4.39: Cocoon shell percentage



4.6.1.4. Percent Good Cocoons

80% of cocoons were categorized as of good quality, reflecting the expected proportion in Control (CN). The percentage dropped to 63.3%, demonstrating a decline in the quality of cocoons in Heat Shock (T1). The percentage rose in ascorbic acid supplemented (T2) group to 90%, emphasizing the improvement in the quality of cocoons and the percentage remained high at 83% heat shock + ascorbic acid supplemented (T3) group, showcasing a maintained improvement in the quality of cocoons.

Figure 4.40: Percent good cocoons



4.6.2. Quality evaluation of silk filaments reeled from the cocoons (post cocoon parameters)

Silk filaments were reeled from the cocoons of four different groups and measured the filament length, weight, size (denier) and reelability percentage for evaluating the quality of cocoon produced. Group T2 (Vitamin C supplemented group) showed better quality of silk filament than the other groups and group T1 (Thermal stress exposed group) showed the least quality of silk filaments. Group T3 (Vitamin C supplemented + Thermal stress exposed group) showed an improvement in the quality of filaments than that of the thermal stress exposed group. Ascorbic acid supplementation demonstrated a positive impact on filament characteristics, especially under heat shock conditions. The results highlight the potential of ascorbic acid in improving filament length, weight, size, reelability, tenacity and elongation, offering insights for optimizing sericulture practices.

Table. 4.18. Quality evaluation of silk filament reeled from the cocoons of control and experimental groups.

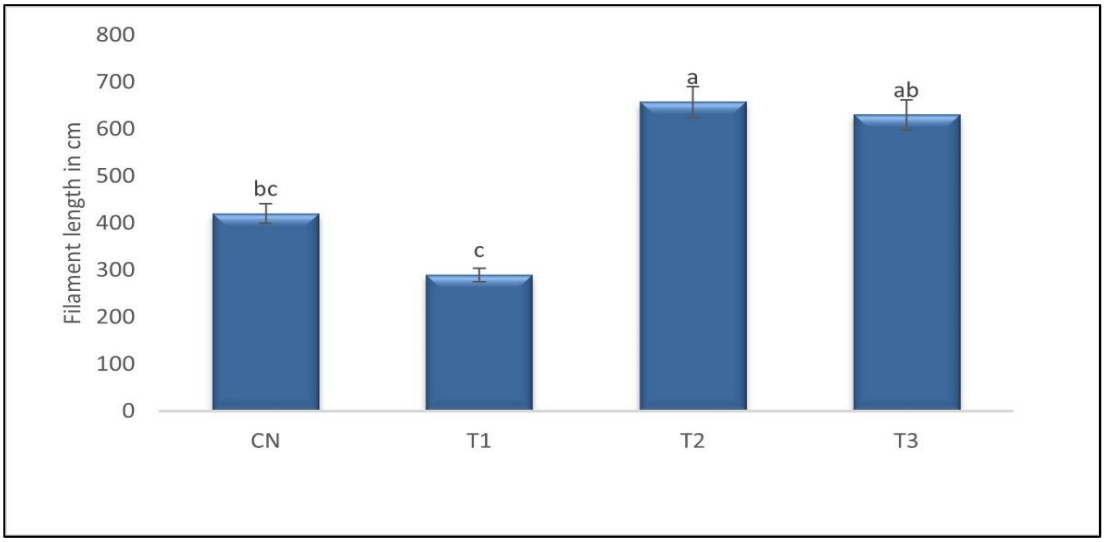
Groups	Filament length (cm)	Filament weight (gm)	Filament size(denier)	Reelability %
CN	420±0.9^{c}	0.16±0.02^{c}	0.34	88%
T1	290±0.52^{d}	0.07±0.01^{d}	0.21	58%
T2	658±0.63^{a}	0.28±0.02^{a}	0.42	90%
T3	630±0.4^{b}	0.21±0.05^{b}	0.35	89%

The variation between the groups was calculated by ANOVA followed by Tukey's test. a, b, c and d are the values differs significantly at $P<0.05$, n=6

4.6.2.1. Filament length (cm)

In terms of filament length, vitamin C supplemented group T2 exhibited the longest filaments with an average length of 658±0.63 cm, followed by T3 (630±0.4 cm), CN (420±0.9 cm), and T1 (290±0.52 cm). This suggests that T2 has the advantage of longer silk filaments compared to the other groups and T1 has the shortest filament length. The filament length significantly increased in the Ascorbic Acid Supplemented (T2) group compared to the Control (CN) and Heat Shock (T1) groups. Although lower than the supplemented group, the Heat Shock + Ascorbic Acid Supplemented (T3) group showed a recovery compared to the Heat Shock (T1) group.

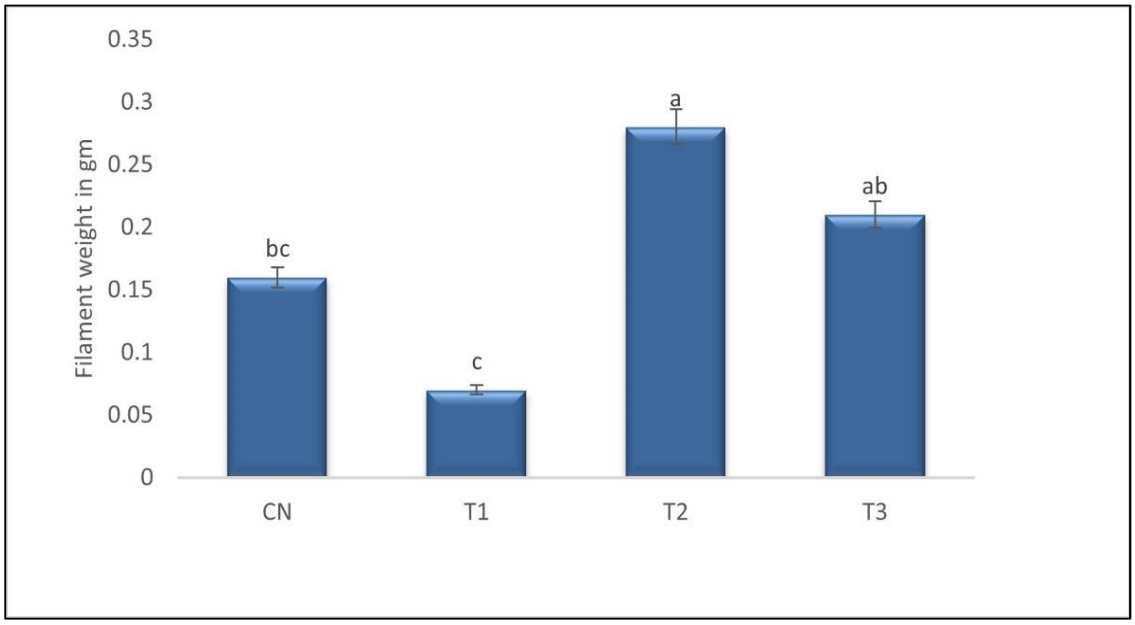
Figure 4.41: Filament length of the cocoon



4.6.2.2. Filament weight (gm)

Filament weight, measured in grams, also varied among the groups. T2 had the highest filament weight at 0.28±0.02 gm, followed by T3 (0.21±0.05 gm), CN (0.16±0.02 gm), and T1 (0.07±0.01 gm). This indicates that T2 produced silk filaments with the greatest mass. The filament weight significantly increased in the vitamin C supplemented (T2) group compared to the thermal stress (T1) and control (CN). The thermal stress + vitamin C supplemented (T3) group demonstrated a positive influence on filament weight even under thermal stress

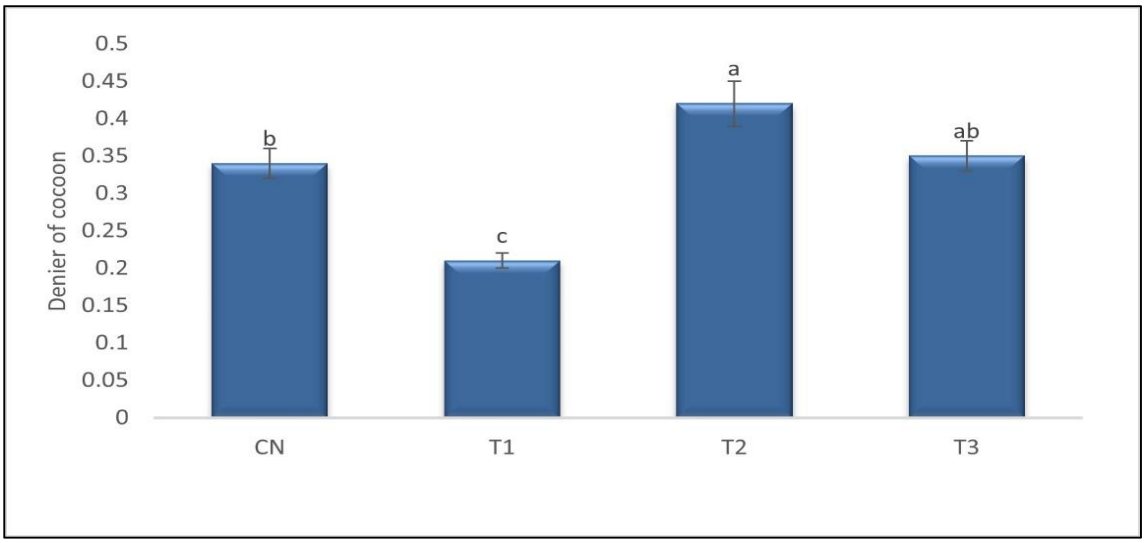
Figure 4.42: Filament weight of the cocoon



4.6.2.3. Filament Size

Filament size, denoted in denier, exhibited a similar trend. T2 had the largest filament size at 0.42 denier, followed by T3 (0.35 denier), CN (0.34 denier), and T1 (0.21 denier). This suggests that T2 produced silk filaments with a larger size compared to the other groups. The filament size (denier) significantly increased in the vitamin C supplemented (T2) group compared to the thermal stress (T1) and control (CN). The thermal stress + vitamin C supplemented (T3) group showed a partial recovery compared to the Heat Shock (T1) group.

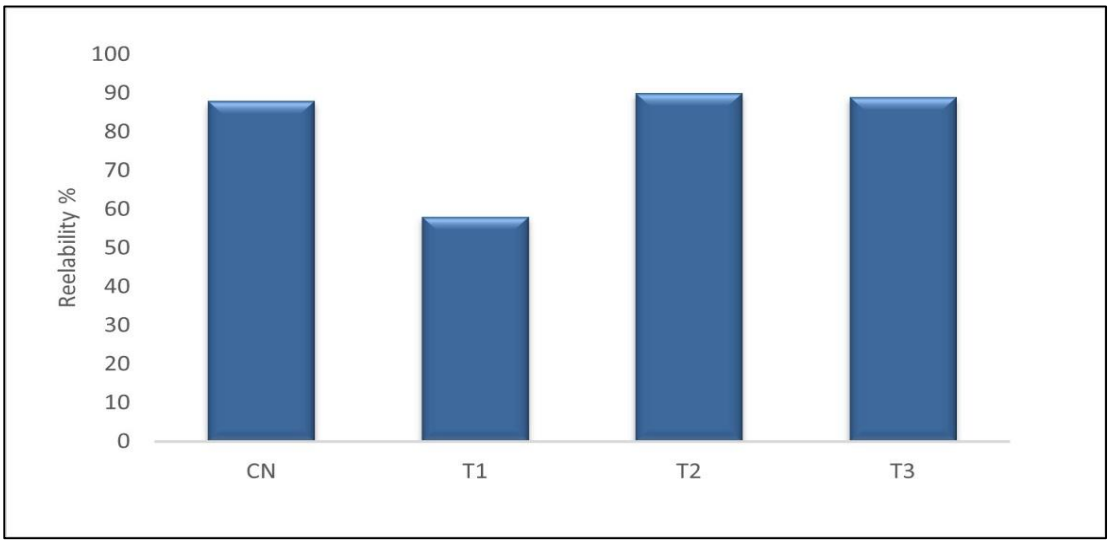
Figure 4.43: Filament size (Denier) of the cocoon



4.6.2.4. Reelability Percentage (%)

Reelability percentage represents the efficiency of reeling silk from the cocoon. T2 demonstrated the highest reelability percentage at 90%, followed by T3 (89%), CN (88%), and T1 (58%). This indicates that T2 had a superior reeling efficiency compared to the other groups. The reelability percentage significantly increased in the Ascorbic Acid Supplemented (T2) group compared to the thermal stress (T1) and control (CN). The thermal stress + vitamin C Supplemented (T3) group showcased a maintained improvement in the reliability of filaments compared to the Heat Shock (T1) group.

Figure 4.44: Reelability percentage of the cocoon



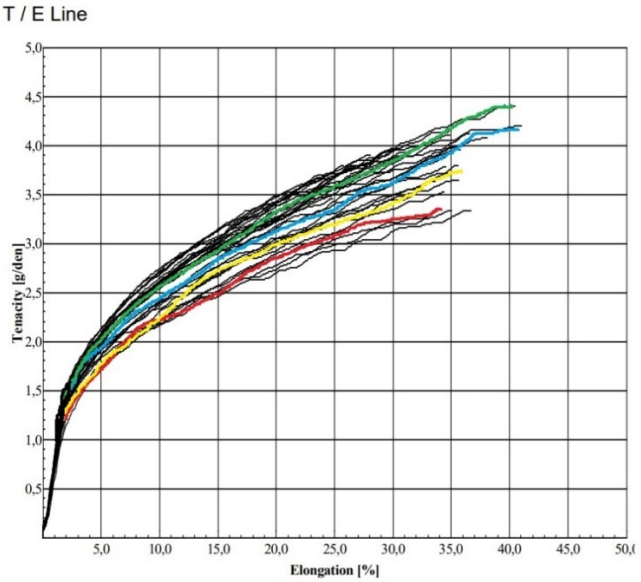
4.6.2.5. Tenacity and Elongation

In our study, we focused on assessing the strength of silk fibers obtained from different groups, with tenacity and elongation serving as key parameters. Tenacity, indicative of silk quality, was analyzed to evaluate cocoon production quality. Results revealed that the vitamin C supplemented group exhibited the highest tenacity (4.1g/den), while the thermal stress-exposed group demonstrated the lowest (3.79g/den) compared to the control. Additionally, vitamin C supplementation under thermal stress enhanced both tenacity and elongation compared to the heat shock group, suggesting improved silk production under adverse conditions. Figure 4.37 illustrates the comparison of average silk filament strength among the four groups using tenacity and elongation line.

Table 4.19: Tenacity and Elongation of silk filament of control and experimental groups.

Silk filament Parameters	Control (CN)	Thermal stress (T1)	Vitamin C supplemented (T2)	Vitamin C + Thermal stress (T3)
Tenacity (g/den)	3.9	3.79	4.1	3.8
CV% of Tenacity	9.1	8.30	9.6	8.41
Elongation %	40.46	32.78	41.5	35.1
CV% of Elongation	16.12	14.83	16.82	15.30

Figure 4.45: Tenacity / Elongation Line of control and treated cocoons.



Graph showing the ratio of tenacity and elongation of silk filament of control and treated groups of fifth instar silkworm larvae.

- Control
- Thermal Stress
- Vitamin C supplemented
- Vitamin C supplemented+ thermal stress.

CHAPTER 5

DISCUSSION

5. Discussion

The farmed silkworm, *Bombyx mori*, is a herbivorous insect and a representative of the order Lepidoptera. Being poikilothermic organisms, silkworms experience a direct impact on their physiological functions with an increase in temperature. Temperature is one of the key abiotic elements that affects the development and cocoon production (Benchamin and Jolly, 1986). High temperatures may eventually reduce the ability of silkworms to generate high-quality cocoons, which will have an impact on silk production (Willmer *et al.,* 2004). The increased temperatures not only affect the silkworm larvae but also the quality of the mulberry leaves, which are the primary source of nutrition for the silkworms. To improve the industry, there is a need to supplement the silkworm feeds with additional nutrients. However, many of the existing supplementation methods are expensive and not affordable for rural farmers. In this study, the potential of fortifying mulberry leaves with vitamin C to overcome the problems caused by thermal stress in silkworms was investigated.

The fifth instar silkworms were chosen for the study as this stage is the most active and important for metabolic and physiological activities. The gut, selected as the experimental organ for the study, holds a vital role in processes such as digestion, nutrition, and absorption. Gut bacteria, present in the cells of the gut, are essential for normal life activities of silkworms, including the production of digestive enzymes and minerals. Overall, this study investigates the potential of vitamin C supplementation in overcoming the challenges posed by thermal stress in silkworms. The analysis includes a comprehensive examination of gut bacteria, protein and digestive enzyme activities, Hsp gene expression, gut histology, and economic parameters to evaluate the impact of vitamin C supplementation on cocoon production under thermal stress.

5.1. Diversity of gut bacteria of silkworm *B. mori*

Gut bacteria have the ability to adapt to changes in the insect's diet by altering their population profiles and inducing necessary enzymes (Kaufman and Klug, 1991; Santo-Domingo *et al.,* 1998). These symbiotic gut bacteria play a crucial role in nutrient provision and digestion, positively influencing the development and fitness of the host (Cleveland, 1923; Fukatsu & Hosokawa, 2002). Additionally, gut bacteria can affect the host's developmental processes through direct interactions with the host, contributing to immunity (Hosokawa *et al.,* 2007) and cellular homeostasis (Kikuchi

et al., 2007) and aiding in adaptation to changing conditions in the gut environment. Thus, the gut microbiota can be considered as a bacterial organ integrated into the host's biological system (Beackhed *et al.,* 2005). The physiology of the host can also be influenced by the gut microbiota, through interaction with the immune system of the host or by providing extra nutrients to retain the host's metabolic equilibrium (Douglas, 2015). The endosymbionts of insects perform a significant role in the host pathogen defence and enhance the nutrition by facilitating the digestion of food (Schmidt and Engel, 2021). According to earlier studies, the gut flora can produce enzymes required for the breakdown of carbohydrates and also influences synthesis of neurotransmitters in the host (Yano *et al.,* 2015; Neuman *et al.,* 2015; Gilmore and Ferretti, 2003; Wexler, 2007). The composition of insect gut flora is closely associated with the environment, nutrition, stage of development and physiological condition (Yun *et al.,* 2014). Extreme temperature fluctuations can modify the gut microbiota of the silkworm *B. mori*, which has an adverse effect on sericulture (Sun *et al.,* 2017). It is reported that, when exposed to high temperatures, the gut microbial composition of silkworms significantly reduced (Sun *et al.,* 2022).

The composition and diversity of gut bacteria in the control and experimental groups were analyzed using 16S rRNA metagenomic sequencing and compared with conventional culture-dependent methods. The dominant bacterial genera found in the silkworm gut included *Bacillus, Lactobacillus, Citrobacter, Pseudomonas, Enterobacter,* and *Klebsiella*. In which *Bacillus* was the common bacteria found in all groups in the culture dependent method. These findings align with previous studies reporting the dominance of these genera in insects (Broderick *et al*., 2004). The 16s rRNA genome sequencing data revealed variations in the bacterial populations between the treatment and control groups. The results revealed that the absolute count and diversity of gut microbes of silkworm larvae were decreased in temperature exposed group than the larvae of control which is consistent with previous report (Sun *et al.,* 2022). Whereas the abundance and diversity of bacteria increased in the group supplemented with vitamin C and in the group supplemented with vitamin C under thermal stress when compared to that of thermal stress group. The presence of diverse bacterial communities in the gut of silkworms has been found to play a crucial role in their overall health and development. Various studies have demonstrated that supplementing the diet with certain compounds, such as tormentic acid and vitamin C,

can positively impact the microbial profile of the silkworm gut. Veysal Bay *et al.,* (2022) found that tormentic acid increased the relative abundance and diversity of bacteria in the gut of *B. mori*. Similarly, Dong *et al.,* (2018) reported that changes in the di*et al*tered the microbial composition and diversity in the gut of silkworms.

Specifically, the supplementation of vitamin C resulted in a decrease in the abundance of bacteria compared to the control group. Though, certain beneficial bacteria, including *Citrobacter, Klebsiella, Erwinia, Pseudomonas, Enterobacter, Clostridium*, and *Enterococcus*, were enhanced by vitamin C supplementation. However, the thermal stress group had a drop in these advantageous bacterial abundances. The decrease seen in the bacterial population among the group supplemented with vitamin C may be due to the prevalence of other advantageous bacterial communities. Certain Firmicutes bacterium species generate antibiotics and enzyme inhibitors that hinder the development of other gut microbiota (Pandey *et al.,* 2013). It is worth noting that the presence of other dominant beneficial bacterial communities may have contributed to the decline in bacterial population in the vitamin C supplemented group. This is consistent with Ivan *et al.,* (2022).

Firmicutes, Bacteroidetes and Proteobacteria are the dominant bacteria present in control and treated groups. The abundance of these bacteria is in agreement with the findings of (Chen *et al.,* 2018,2020, Dong *et al.,* 2018, Sun *et al.,* 2022). The presence of Proteobacteria symbionts in both control and treated groups was found to aid in the degradation of polymers in mulberry leaves and provide nutrients to the silkworm hosts (Suen *et al.,* 2010). In the current study, the percentage of Bacteroidetes showed a significant decrease in the temperature-treated sample (29.08%) compared to the control (30.48%), while it increased in the other two vitamin C-supplemented groups (T2: 31.67% and T3: 32%). These bacteroidetes are important for the production of fatty acids which are used as an energy source for the host (Krajmalnik-Brown *et al.,* 2012). These symbionts play a significant role in energy production, which may be the reason for the increase in larval length and the production of good quality cocoons in the vitamin C supplemented group.

The abundance of clostridia within the Firmicutes phylum was observed in all groups, with variations noted. Specifically, the percentage of Clostridium was lower in the temperature-treated sample and higher in the vitamin C-treated group compared to the

control. A similar finding revealed that a significant decrease in the abundance of Clostridia after the exposure of temperature also affects the digestion of carbohydrates and short chain fatty acids of the host (Sun *et al.,* 2022). Clostridium cluster IV, IX and XIVa can be used for the fermentation of dietary products includes butyric acid, acetic acid, formic acid and propionate which involves in the metabolic functions of host propionate (Louis *et al.,* 2007; Eckburg *et al.,* 2005; Ratajczak *et al.,* 2019; Wolever *et al.,* 1989). Some bacteria may be sensitive to temperature and that might be one of the reasons for the reduction in the number of gut bacteria in temperature treated samples (Sun *et al.,* 2017).

Reports showed that exposure to high temperature may lead structural damage to gut tissues that intern adversely affect the symbiotic microbiota under thermal stress (Fast and Angus, 1965; Koch *et al.,* 2019a). Sun *et al.,* (2022) opined that when the fifth instar larvae exposed to high temperature reduces the growth and development of silkworm by altering the intestinal microbiota and also showed a reduction in the activity of digestive enzymes. In the present study, a significant decrease in the activity of digestive enzymes, including lipases, proteases, amylases, and cellulases, was observed in the heat shock-treated group compared to the control. The insect intestinal flora is supposed to be involved in the breakdown and absorption of food (Wong *et al.,* 2014). Proteases secreted by *Cyanobacteria* and *Stenotrophomonas* in the gut of silkworm helps in the digestion and absorption of chlorophyll and other components present in the mulberry leaves (Sun *et al.,* 2016; Wang *et al.,* 2016). Certain bacterial strains, such as *Acinetobacter, Sphingomonas*, and *Pseudomonas*, have been found to have enzymatic activity related to the breakdown of carbohydrates, lipids, and cellulose in the silkworm gut (Prem *et al.,* 2010). The observed decline in enzyme activity has implications for the digestive process and nutrient absorption. This could be a contributing factor to the diminished growth of larvae and cocoons, as evidenced by the reduction in morphometric parameters in the present study.

The gut microbiota not only contributes to the host through enzyme production but also provides nutrition. Bacteria can be a food source for the host, as they are considered single cell proteins. Additionally, bacteria can produce various vitamins, such as B-group, C, E, A, and coenzymes (Shimizu, 2008). Firmicutes bacteria have been shown to assist the host in tolerating extreme conditions, harvesting energy from

food, and enhancing immunity. The symbiotic association of Firmicutes with the gut contributes to increased energy harvest from the diet and may explain the increase in gut and larval weights in silkworms treated with vitamin C.

The decrease in the abundance of beneficial bacteria in the thermal stress group and their increase in the vitamin C supplemented group may have contributed to the enzyme activity and protein concentration in silkworm larvae. This higher level of activity in the vitamin C supplemented group may have led to a better cocooning process and improved silk quality. On the other hand, the decline in beneficial bacteria in the thermal stress group may have resulted in lower larval and cocoon quantity and quality due to their role in enzyme production and nutrient provision. Our findings underscore the critical role of gut bacteria in normal development and the production of healthy cocoons. However, environmental changes can disrupt the intestinal flora, potentially impacting the usual development and quality of the produced cocoons. But vitamin C supplementation may have the potential to overcome the thermal stress to balance the adverse effect. In short, these studies highlight the importance of a diverse gut microbiota in the health and development of silkworms. Supplementation with compounds like vitamin C can enhance the microbial profile and have a positive impact on various physiological processes in silkworms.

5.2. Morphometric parameters of Silkworm larvae

The success of cocoon production in sericulture relies heavily on the healthy growth of silkworm larvae (Islam *et al.,* 2004). During their larval stage, silkworms consume mulberry leaves to produce abundant silk thread, and the quantity and quality of the resulting cocoon are directly influenced by larval growth. The economic significance of the cocoon is determined by the growth parameters of the larvae, making it a pivotal factor in sericulture. Studies by Rahmathulla *et al.,* (2004 & 2013) highlighted those optimal environmental conditions, specifically at 25°C and 70% relative humidity (RH), contribute to significantly greater growth and development of silkworm larvae and silk glands. Shirota, (1992) further emphasized the sensitivity of silkworms to high temperatures during the fourth and fifth stages of development. Nutritional quality of food is identified as another critical factor influencing larval growth and insect development physiology (Murugan and George, 1992).

Additionally, research by Kanafi, (2007) demonstrated the positive impact of various vitamins, including vitamin C, on the nutritional enrichment of mulberry leaves, ultimately enhancing the growth and development of *Bombyx mori*.

In the current study, our focus was on analyzing the role of vitamin C in the growth and development of fifth instar silkworm larvae under thermal stress. The results revealed a substantial decrease in the periodic length and weight gain of larvae exposed to thermal stress. Notably, vitamin C supplementation led to a significant increase in morphometric parameters when compared to both the control and other experimental groups. Even under thermal stress, larvae supplemented with vitamin C exhibited notable improvements in growth metrics. This accelerated growth and development, in turn, altered the characteristics of the resulting cocoon, aligning with findings by Rahmathulla and Suresh, (2013). Our observations indicated a significant decrease in the length and weight of larvae in the thermal stress group compared to the control and other experimental groups, consistent with the findings of Devi and Karuna, (2012). Their studies showed a direct adverse impact on the health of silkworms when exposed to temperatures above 30°C. Similarly, Omana and Karumathil, (1995) noted that temperatures exceeding 43°C could be lethal to all developmental stages of certain strains of *Bombyx mori*, further underscoring the negative effect of temperature on larval length and weight.

Vitamin C supplementation emerged as a significant influence of larval growth and development. Under temperature stress, the experimental group, which received vitamin C supplementation, exhibited elevated growth and yield attributes compared to the temperature stress-exposed group. These findings align with studies by Sengupta *et al.,* (1972) and Haq and Saleem, (1985), who reported enhanced morphometric parameters with vitamin C and nitrogen supplementation, respectively. The positive association between food intake and larval weight, as observed by Horie & Watanabe, (1983), are also in agreement with our findings. In conclusion, our observations indicate that silkworms respond positively to mulberry leaves supplemented with vitamin C when exposed to heat stress, leading to an improvement in cocoon and silk quality.

5.3. Biochemical Analysis

Having an understanding of the gut enzymes and protein composition of the silkworm gut is crucial for creating new lepidopteran tactics. In insects, the larval gut serves as the initial site where complex food molecules are broken down by digestive enzymes before being utilized. In order to effectively transform the organic food molecules in the leaf into usable macromolecules, the silkworm larval enzyme system is essential. The analysis of an insect's protein profile and enzyme activity is crucial for understanding several physiological processes. So, in this study the protein and enzymatic response of the silkworm gut was therefore examined using fifth instar larvae.

The successful growing of silkworms and the production of high-quality silk are directly influenced by a variety of environmental and biological factors. The temperature has a critical role in embryonic and postembryonic development and it determines the fate of the silkworm, *B. mori* L. (Dinesh *et al.,* 2012). In the current study, a negative impact of temperature was observed. Larvae subjected to temperature exhibited reduced levels of proteins and enzyme activity. The nutritional value of the diet was enhanced when additional nutrients or fortification agents were introduced, making the diet more beneficial from a nutritional standpoint (Bajpeyi *et al.,* 1991). In the present study, the rate of metabolism of silkworm was accelerated when supplementing the diet with 0.2% vitamin C by averting the oxidative damage generated by the exposure of high temperature.

5.3.1. Concentration of protein present in the silkworm gut

The investigation into the total soluble protein content in the gut of fifth instar larvae yielded insightful results in our study. Notably, the gut protein content significantly increased in the larval group supplemented with vitamin C, presenting a marked contrast to the control group. Conversely, exposure to high temperature (40±2°C) resulted in a decrease in gut protein content in larvae. However, the introduction of vitamin C supplementation under heat shock conditions led to a considerable upsurge in protein content compared to both the control and stress-exposed groups.

These findings suggest that larvae receiving vitamin C supplementation exhibit heightened protein metabolism and greater physiological activity under heat stress conditions. Similar observations were made by Manoharan (1997), who noted

increased protein quantity in the silkworm intestine of *B. mori* when hydrolyzed soybean was added. This aligns with our results and underscores the impact of external factors, such as vitamin C, on protein levels in the silkworm gut. Consistent feeding activity emerges as a major factor influencing protein content following the fourth moult, as noted by Krishnaswamy (1978). Further supporting our findings, Quraiza *et al.,* (2008) observed significant increases in protein content in various silkworm tissues, including the silk gland, fat body, and muscles, when fed with 1% and 2% vitamin C. Additionally, fortifying mulberry leaves with herbal extract and feeding fifth instar larvae resulted in a significant improvement in protein levels and enzymatic reactions catalyzed by protease and amylase (Vitthalrao and Sucheta, 2012).

Temperature fluctuations, as highlighted by Rehab (2013), can interfere with protein activities, affecting processes like egg formation and oogenesis. In our study, the administration of vitamin C played a crucial role in enhancing protein levels in the silkworm's intestine, emphasizing its potential role in modulating protein content. The impact of pH on protein functions and the structural alterations induced by heat, destabilizing weak interactions in proteins, nucleic acids, and membranes, are crucial factors as reported by Hochachka and Sommero (1973). Exposure to high temperatures may disrupt or halt normal protein synthesis, as observed in our study where the protein level of heat shock-exposed larvae decreased compared to the control group. These results align with the findings of Sreekumar *et al.,* (2007).

Our study highlights the dynamic influence of vitamin C supplementation on protein metabolism in silkworm larvae, especially under thermal stress conditions. The findings contribute to our understanding of the relationship between environmental factors, nutritional supplements, and protein dynamics in the context of sericulture.

5.3.2. Activity of Amylase

Since enzymes are proteins, they are known to be vulnerable to damage from radiation, chemicals, and extreme temperatures. The performance of the larvae in effectively converting the organic food molecules of the leaf into usable biomolecules is greatly influenced by the enzyme system. Depending on the kind of enzyme, temperature, mutagen exposure, and the amount of time between treatment and measurement, the activity of the enzyme may change (Lakshmi & Ananthanarayana,

1997) The highest amylase activity was found in group T2 (vitamin C supplemented) and the lowest in group T1(heat shock), suggesting that the addition of vitamin C may have accelerated metabolism and increased enzyme production, which in turn may have enhanced the activity. On the contrary, the lower enzyme activity might be due to the direct exposure of temperature which alters the metabolism to a high degree. Group T3 showed a significant increase in the enzyme activity than group T1 which proved that supplementation of vitamin C has the ability to overcome the damage caused by heat shock. Similarly, addition of horse gram flour at the optimal concentration boosted the activity of the amylase enzyme in silkworm larvae, followed by corn flour at the optimal substrate concentrations of 2.5% and 2.7%, respectively. (Nijagal and Kumara, 2017).

The observed fluctuations in amylase activity in the present study can be linked to the presence of amylase-producing bacteria in the gut of the larvae. A compelling theory suggests that the higher amylase activity in the ascorbic acid supplemented group is probably due to an increased population of bacteria that produce amylase, whereas the lower amylase activity in the group under thermal stress might be the result of a decreased production of these beneficial bacteria. Corroborating our findings, the study conducted by Liang *et al*., (2015) sheds light on the amylase-generating capabilities of *Enterococcus* and *Erwinia* within the silkworm gut. This aligns seamlessly with our results, which indicate an increase in the abundance of *Enterococcus* and *Erwinia* in both the vitamin C-supplemented group and the group subjected to thermal stress with vitamin C supplementation. Conversely, a decrease in their numbers is noted in the thermal stress-exposed group when compared to the control. These findings underscore the pivotal role of gut bacteria, specifically Enterococcus and Erwinia, in influencing amylase activity. The positive correlation between the presence of these bacteria and enhanced amylase activity in the vitamin C-supplemented groups further emphasizes the symbiotic relationship between gut bacteria and enzymatic processes crucial for digestion.

In essence, our study not only contributes to understanding the factors influencing amylase activity in silkworm larvae but also accentuates the importance of gut bacteria in this digestive pathway. The modulation of amylase activity under varying conditions reflects the intricate balance within the silkworm gut microbiome, with potential implications for cocoon production and quality. This recognition of the

interplay between gut bacteria, amylase production, and external factors provides a foundation for refining sericulture practices and optimizing the nutritional well-being of silkworms.

5.3.3. Activity of Cellulase

Feeding on mulberry leaves, insects can host diverse gut microbial communities engaged in cellulose degradation, a phenomenon well-documented in several reports (Kaufman & Klug, 1991; Prem *et al.,* 2010). Although cellulose represents a rich carbon source, its crystalline or amorphous microfibrillar structure in plant cell walls makes it less accessible to the host organism (Watanabe & Tokuda, 2010). The complex process of breaking down cellulose fibers into simpler sugar residues primarily involves the participation of bacteria within the insect gut (Warnecke *et al.,* 2007)

In the context of our study, cellulase enzyme activity exhibited a notable decrease in the thermal stress group compared to both the control and the other experimental groups. Remarkably, the vitamin C-supplemented group displayed elevated cellulase enzyme activity, and even under conditions of thermal stress, vitamin C supplementation resulted in increased cellulase enzyme activity in silkworm larvae compared to the control and the heat shock group. The observed reduction in enzyme activity in the thermal stress group may be attributed to a decline in the population of enzyme-producing bacteria, while the rise in activity in the vitamin C-supplemented group could be linked to an increase in the bacterial community. The pivotal role of gut bacteria in the production of cellulase-producing bacteria is supported by the findings of Prem *et al*. (2010). Their research unveiled the presence of bacterial species capable of degrading cellulose, a crucial component of the silkworm *B. mori* diet. The identified bacterial species, including *Bacillus sp., Proteus vulgaris, Klebsiella sp., Citrobacter freundii*, and *Pseudomonas sp*., were found to be proficient in breaking down cellulose. Furthermore, the study highlighted an intriguing trend wherein the quantity of cellulolytic bacteria increased with each instar.

Building upon the complicated dynamics of cellulose degradation, it is crucial to develop deeper into the specific bacterial communities associated with this process. In our study, an intriguing observation emerges as we explore the presence of key cellulolytic bacteria, including *Bacillus, Klebsiella, Citrobacter,* and *Pseudomonas*.

The abundance of these bacteria is notably influenced by external factors, particularly thermal stress and vitamin C supplementation. In the thermal stress group, there is a discernible reduction in the presence of *Bacillus, Klebsiella, Citrobacter*, and *Pseudomonas* compared to other groups. This decrease aligns with the observed decline in cellulase enzyme activity in the same group, suggesting a potential correlation between bacterial abundance and enzyme production. The diminution in these cellulolytic bacteria in the thermal stress group may contribute to the decreased cellulase activity, emphasizing the integral role these bacteria play in cellulose digestion. Conversely, in the vitamin C-supplemented group, there is a notable increase in the presence of *Bacillus*, *Klebsiella, Citrobacter,* and *Pseudomonas*. This rise is concurrent with the enhanced cellulase enzyme activity observed in the same group. The heightened abundance of cellulolytic bacteria in the presence of vitamin C supplementation aligns with the notion that these bacteria play a pivotal role in the breakdown of cellulose into simpler sugar residues.

5.3.4. Activity of Lipase

Lipids play a pivotal role in the digestion and metabolism of silkworms (*Bombyx mori*), as highlighted by recent research. Studies such as Zhang, (2018) emphasize the significance of lipase enzymes in breaking down complex lipids present in the mulberry leaves, the primary diet of silkworms. Lipids, acting as a major energy source, also contribute to silk protein synthesis during the pupal stage, influencing cocoon formation. Understanding the importance of lipids in silkworm digestion is not only fundamental for unraveling their physiological processes but also holds implications for optimizing sericulture practices to enhance silk production and overall larval health.

The investigation into lipase activity in our study reveals a notable contrast between the thermal stress and vitamin C-supplemented groups. The thermal stress group exhibits lower lipase enzyme activity, while the vitamin C-supplemented group demonstrates elevated activity. This intriguing observation aligns with the findings of Prem *et al.,* (2010), who identified specific bacterial strains, including *Acinetobacter, Sphingomonas*, and *Pseudomonas*, with enzymatic activity related to lipid breakdown in the silkworm gut. The abundance of these lipase-producing bacterial strains appears to correspond with the observed variations in lipase activity. The thermal stress group,

characterized by lower lipase activity, shows a decrease in the population of these lipid-degrading bacteria, while the vitamin C-supplemented group exhibits a higher abundance. Even under conditions of heat shock, the vitamin C-supplemented group maintains elevated lipase activity, suggesting a potential modulation of lipase-producing gut microbes by vitamin C.

5.3.5. Activity of Protease

One of the essential enzymes that aids in the conversion of mulberry protein to silk protein in *B. mori* (L) is the gut protease enzyme. Protease are crucial digestive enzymes that are synthesised more quickly in the silkworm larval stage (Lakshmi & Ananthanarayana, 1997) and plays a significant role in the digestion of proteins in the insect digestive systems (Abudabos, 2012). In our study, it was observed that the amount of protease activity in the gut of larvae showed higher than that of other gut enzymes. This could be for the conversion of mulberry proteins into better quantity of silk proteins. Protease activity was found to be higher in group T2 and significantly lower in the group T1. The reduction in protease activity observed in our study may be due to the diminished abundance of bacteria responsible for protease production. Certain bacteria possess the capability to synthesize enzymes that facilitate the breakdown of proteins. This aligns with findings reported by Sun *et al.,* (2016) and Wang *et al.,* (2016), who documented the role of proteases secreted by *Cyanobacteria* and *Stenotrophomonas* in the silkworm gut. These enzymes are crucial in the digestion and absorption processes of chlorophyll and other components found in mulberry leaves. Notably, our observations indicate that the thermal stress group exhibited a lower bacterial population, which correlates with the observed decrease in protease activity. In contrast, larvae supplemented with vitamin C under thermal stress demonstrated a significant increase in bacterial abundance. This augmentation in bacterial numbers in the presence of vitamin C likely contributes to the higher protease activity observed in this group.

These findings underscore the intricate relationship between bacterial abundance, protease activity, and the influence of external factors such as thermal stress and vitamin C supplementation. The role of bacteria in enzyme production within the silkworm gut emerges as a critical factor in understanding the digestive processes and metabolic responses under different environmental conditions. Further exploration into the specific bacterial species involved and their interactions with thermal stress

and nutritional supplementation could provide valuable insights into optimizing silkworm health and productivity.

5.4. mRNA associated stress protein expression (Hsps)

Heat shock proteins were initially identified in insects (Ritossa, 1962) and are essential for surviving acute thermal stress as well as other types of stress (Sorensen *et al.,* 2019). Primarily, they serve as molecular chaperones, preventing proteins becoming denatured under adverse conditions. Hsp's chaperone action is based on the molecular mechanism by which this protein binds to misfolded or denatured proteins to stop them from aggregating and causing damage to cells (Feder and Hofmann, 1999). Naturally, the intron region was absent from several heat shock protein genes (Hsp 70, Hsp 90, or Hsp 20.4). Primers were designed for the initial exonic region of Hsp 40, which comprises two exons and an intervening intronic sequence. The longest exon without an intron is the most noticeable feature of the Hsp gene locus. Since heat shock inhibits the splicing process, thermal stress-related proteins usually lack splicing mechanisms (Yost and Lindquist, 1986).

So, analysis of the gene expression pattern of Hsp 20.4, Hsp 40, Hsp 70 and Hsp 90 of silkworm larval gut were studied and compared in the experimental groups with the control. The guts of silkworm larvae in their fifth instar expressed all four of the Hsp genes. But variation in the expression level was observed in each experimental group. Compared to the control group, the heat shock exposed group (T1) and the heat shock + vitamin C supplemented group (T3) both had higher levels of Hsp expression. According to the findings, each of these Hsp genes functions as a molecular chaperone during a heat shock and is implicated in thermal stress tolerance. These Heat Shock Proteins (Hsps) serve the function of defending and safeguarding proteins from heat-induced denaturation and other stressful conditions. Vasudha *et al.,* (2006) carried out similar study to determine the connection between heat shock, Hsp expression, and *B. mori*'s commercial features. The observed result suggests that upregulating the expression of the Hsp gene has a significant effect on an organism's thermotolerance and ought to be one of its adaptive responses to heat stress.

5.4.1. Expression of Hsp 20.4

Hsp 20.4 belongs to the family of genes called small heat shock proteins (sHsp or Hsps). During heat shock, these proteins form an oligomeric complex, and the effective functioning of chaperones depends on the disaggregation of this complex (Haslbeck *et al.,* 1999). *B. mori* possess the highest number of insects sHsp genes documented to date, 16 in total (Li *et al.,* 2009b). These proteins play an integral part in apoptosis and heat stress tolerance (Arrigo, 2005; Feder and Hofmann, 1999). Six genes were identified in *B. mori* strain P50 that encode sHsp19.9, sHsp20.1, sHsp20.4, sHsp20.8, sHsp21.4, and sHsp23.7 (Sakano *et al.,* 2006). According to Sun and Mac Rae (2005), sHsps bind partly denatured proteins to stop irreversible protein aggregation under stress.

Our findings suggest that, in comparison to the control, there was an increase in the expression of gene Hsp 20.4 in the gut of larvae under heat stress conditions. Supplementation of vitamin C alone shows a down regulation in the gene expression levels compared to the control and other groups. The expression of the Hsp 20.4 gene was induced in the gut of the larvae supplemented with vitamin C under thermal stress condition. Li *et al.,* (2012) also reported on the increase of Hsp 20.4 gene expression during heat stress. Additionally, Sakano *et al.,* (2006) observed that when the *B. mori* strain p50 was subjected to heat shock, the sHsp gene expression level was upregulated. The experimental group supplemented with vitamin C under thermal stress showed the highest level of gene expression and this may be because vitamin C has antioxidant action and the highest degree of thermotolerance.

5.4.2. Expression of Hsp 40

Hsp 40 is a co-chaperone DNA J-like protein that plays a crucial role in protein folding with Hsp 70 after heat shock (Morimoto *et al.,* 1990). In our study, we observed a significant increase in Hsp 40 expression in the gut of larvae exposed to heat shock, while the vitamin C alone supplemented group reduced its expression. Similarly, previous research has shown higher expression of Hsp 40 in thermotolerant strain Nistari silkworms (Velu *et al.,* 2008) and in resistant silkworms after viral infection (Lekha *et al.,* 2014). Interestingly, the group supplemented with vitamin C under thermal stress exhibited the highest expression of Hsp 40, possibly due to the mitigating effects of vitamin C on heat-induced cellular responses. The up-regulation

of Hsp 40 genes indicates their important role in thermotolerance in silkworms, consistent with previous studies (Carmel *et al.,* 2011). Overall, these findings suggest that Hsp 40 genes contribute significantly to the adaptation of silkworms to high temperatures.

5.4.3. Expression of Hsp 70

Heat shock proteins 70 are members of a widely distributed family of molecular chaperones that are involved in protein refolding after a stress injury and play crucial functions in protein folding (Gupta and Singh, 1994; Kiang and Tsokos, 1998). All of the gut tissue from the treatment and control groups expressed Hsp 70 gene. The group supplemented with vitamin C under heat stress had considerably increased Hsp 70 expressions, whereas the group supplemented with vitamin C alone showed lower expression. In other insects such as *Heliothis armigera* have also shown variations in Hsp expression in their tissues (Singh and Lakhotia, 2000). Similar findings were reported in *B. mori* also indicating that Hsp 70 was elevated at high temperatures of 37°C and 42°C (Fang *et al.,* 2021). These findings led us to conclude that Hsp 70 plays a significant role in heat tolerance. High mRNA levels of Hsp 70 are induced in vitamin C supplemented under thermal stress group, and this could protect the larvae from thermal inactivation and feeding inhibition. Feder and Krebs (1998) also observed similar observations.

The increase in Hsp 70 gene expression is positively correlated with thermotolerance, as observed in various organisms including lizards (Gehring & Wehner, 1995; Ul'masov *et al.,* 1992). Vitamin C supplementation enhances thermotolerant ability and is associated with the expression of Hsp 70 genes. This may be due to the antioxidant activity of vitamin C, providing cytoprotective effects against oxidative stress. Hsp 70 also exhibits antioxidant activity, and its expression could coincide with increased antioxidant enzyme activity (Creagh *et al.,* 2000 and Sreedhar *et al.,* 2004). The production of Hsp 70 is induced by vitamin C, facilitating thermotolerance in silkworm larvae under heat stress.

5.4.4. Expression of Hsp 90

Unlike in vertebrates, the Hsp90 gene in silkworms and fall armyworms does not contain introns, making it a unique gene in these species (Landais *et al.,* 2001).

Hsp90, along with Hsp70, plays a critical role in helping organisms cope with internal and external stresses. It is constitutively expressed and involved in protein folding (Welch & Feramisco, 1982) and peptide translocation (Brugg *et al.,* 1981) with the assistance of co-chaperones. Recent studies have highlighted the importance of Hsp90 in normal growth and development as well as its connection to ecological and evolutionary relevance (Sorensen *et al.,* 2003; Sorensen and Loeschcke 2007). The present study found an upregulation of Hsp90 gene expression in the gut of silkworm larvae exposed to thermal stress and supplemented with vitamin C. However, vitamin C supplementation alone resulted in a decrease in Hsp90 production compared to other experimental groups. The variation in the transcript level of Hsp 90 was also reported in the silkworm on NPV infection (Lekha *et al.,* 2014). This suggests that Hsp90 may play a role in immune activity during thermal stress or infection.

The Hsp proteins also serve as danger signals to protect the immune system and cytoplasm components from various stresses. Vitamin C supplementation has a functional association with the antioxidant defence system under thermal stress in silkworms and may attenuate heat stress-induced cellular responses. The total concentration of Hsps and their localization within the cell are crucial for acquiring thermotolerance (Kampinga, 1993). The higher expression of Hsps in silkworms supplemented with vitamin C under heat stress is considered a desirable feature for conferring thermotolerance. Comprehending the molecular mechanisms underlying temperature and stress adaptability is crucial for the sustainability of the sericulture industry and the breeding of thermotolerant silkworms.

5.5. Histological Analysis of silkworm gut

The gut, as a central organ for digestion and nutrient absorption, plays a pivotal role in the growth, development, and overall health of organisms. The gut not only serves as the primary site for the breakdown of ingested food but also houses a complex community of symbiotic bacteria, influencing the nutritional ecology of the silkworm. Histological studies provide a detailed insight into the cellular composition, structural organization, and functional morphology of the gut. In the context of silkworms, these studies are requisite for unraveling the relationship between gut histology, digestive processes, and the role of symbiotic microorganisms residing within. The present study shed light on how environmental factors, dietary supplements, or stressors may

impact the gut structure and, consequently, the overall well-being of the silkworm larvae. By examining the histological changes in response to various experimental conditions, we aim to enhance our understanding of the gut's adaptability and resilience.

In our study, the histological analysis of the silkworm larval gut provides valuable insights into the impact of high temperature and vitamin C supplementation on gut morphology. In the control group, the histological features revealed a normal and healthy gut structure characterized by intact epithelial cells, goblet cells, and stem cells. The continuous basal lamina underlying and supporting the epithelial cells indicated a stable and functional gut environment. Contrastingly, the histomorphology of the silkworm larvae exposed to high temperatures displayed various pathological changes. Notably, the fusion of epithelial cells and the widening of goblet cells, along with the accumulation of secretory granules, pointed towards cellular stress and damage. This is consistent with previous results highlighting the damages and structural alterations of the silkworm midgut to thermal stress (Sun *et al*., 2022). A similar study was also reported by Fast and Angus (1965), suggested that high temperatures could induce damage to silkworm intestinal tissue, potentially affecting the permeability of the intestinal wall. This aligns with our speculation that the observed changes in midgut morphology may be linked to damage in the silkworm gut epithelium under high-temperature conditions. The disruption in the arrangement of cells observed in the histological results could be indicative of this temperature-induced damage. The findings of Koch *et al.*, (2019a) also supports the concept that increased temperatures can harm and cause damage to the structure and functioning of the intestines. Interestingly, despite these cellular alterations, the basal lamina and stem cells remained intact, suggesting a potential resilience of the underlying support structure.

The supplementation of vitamin C maintained the integrity of the gut epithelium, as evidenced by the presence of intact epithelial cells, goblet cells, and stem cells supported by a continuous basal lamina. This observation suggests a protective role of vitamin C in preserving the normal histological features of the gut under stressed conditions. In the case of silkworm larvae subjected to thermal stress with vitamin C supplementation, a promising restorative effect was observed. The gut epithelium exhibited signs of recovery, with goblet cells returning to their normal

histomorphological features. The reorganization of epithelial cells to their normal architecture indicated a beneficial impact of vitamin C supplementation in mitigating the adverse effects of thermal stress on gut morphology. Despite the reduced width of goblet cells, their increased secretory activity suggests an adaptive response to the stressor.

In conclusion, the histological results highlight the susceptibility of the silkworm larvae gut to high temperatures, the protective role of vitamin C supplementation, and the potential for recovery under thermal stress conditions when supplemented with vitamin C. These findings contribute to a better understanding of the physiological responses of silkworms to environmental stressors and underscore the importance of nutritional interventions in supporting gut health.

5.6. Quantity and Quality evaluation of cocoons

The cocoon, as the final product in the practice of sericulture, holds immense economic significance, serving as the primary revenue generator for silk producers and the broader sericulture industry. The central objective of silkworm breeding is the continuous improvement of economic traits to enhance profitability. This pursuit becomes particularly crucial when introducing bivoltine races in tropical regions like India, where the stability of cocoon crops under high temperatures is imperative (Datta *et al.,* 1996; Rehab, 2013). The nutritional quality of mulberry leaves plays a pivotal role in influencing the formation of healthier cocoons, making it a focal point of research in the present study. Specifically, we investigate the supplementation of ascorbic acid to enhance the efficiency of larvae under thermal stress, a novel approach with potential implications for the economic viability of sericulture.

The various parameters studied encompassing cocoon characteristics include quantity evaluation of cocoon such as length, weight, width and post cocoon parameters such as length, weight, denier, tenacity and elongation of silk filaments. These parameters are critical indicators of cocoon quality, and their analysis provides valuable insights into the impact of ascorbic acid supplementation on the economic traits of sericulture. Our findings reveal a substantial decrease in all cocoon parameters in the temperature-treated group compared to the control. This decline is a significant concern as it directly correlates with reduced cocoon yield and compromised quality. Potential explanations for these observations include decreased pathogen resistance (Sugnana *et*

al., 2011) and adverse effects on the immune system under thermal stress (Yun *et al*., 2014; Round and Mazmanian, 2009; Belkaid and Hand, 2014). Environmental factors, particularly temperature, have long been recognized as influential determinants of cocoon production.

The silk conversion rate, a crucial economic parameter, emerges as positively influenced by the supplementation of ascorbic acid in silkworm feed (Sarker *et al.,* 1995). The antioxidant activity of ascorbic acid and the induction of heat shock proteins during the larval stage contribute to improved cocoon characteristics, including increased cocoon and shell weight, cocoon length and width, filament length, weight, size, and tensile strength (Das and Medda, 1988). Notably, the study reveals that the increased cocoon shell weight through ascorbic acid supplementation contributes to a more reliable silk filament end product. This finding is in line with the broader objective of improving the efficiency of converting dietary food into the cocoon shell, resulting in elevated denier and cocoon yields. In comparison, the study indicates that 0.2% ascorbic acid supplementation is more effective than other concentrations. This finding aligns with prior research emphasizing the positive impact of vitamin supplementation on cocoon shell weight (Etebari *et al*., 2004).

The evaluation of cocoon quality was conducted by assessing various parameters, including silk filament length, filament weight, filament size (denier), reelability percentage, and tensile strength. Silk filament length plays a crucial role in determining the rate of silk production, with longer filaments generally associated with higher efficiency (Lee, 1999). In this study, Vitamin C supplementation resulted in cocoons with a significantly longer silk filament length of 658±0.63 cm, outstanding in other experimental groups, including the control group with only 420±0.9cm of filament length. The group subjected to temperature treatment exhibited a reduced filament length of 290±0.52 cm. However, the group that received vitamin C supplementation under thermal stress demonstrated an improvement, showing an increased filament length of 630±0.4 cm compared to the thermal stress exposed group. This suggests that the addition of vitamin C under thermal stress conditions may have a positive impact on silk filament length, potentially mitigating the adverse effects of the elevated temperature on cocoon production. The length of silk filaments is influenced by factors such as uniformity in silk secretion and reeling efficiency (Nguku *et al*., 2007).

The parameters of tenacity and elongation play crucial roles in determining the quality and usability of silk fibers. Tenacity refers to the strength of the silk filament, while elongation measures its ability to stretch without breaking. The study indicates variations in tenacity among different groups. The highest tenacity was reported in the vitamin C supplemented group (4.1 g/den), while the thermal stress-exposed group exhibited the lowest tenacity (3.79 g/den) compared to the control. The coefficient of variation for tenacity shows the variability within each group. Among the groups, the Vitamin C supplemented group (T2) had the highest CV%, suggesting relatively higher variability in tenacity compared to the other groups. Koch *et al*., (2019b) highlighted a similar result that high temperatures can affect the activity of related enzymes, possibly influencing silk tenacity.

Elongation also varied across groups, with the vitamin C supplemented group showing the highest elongation (41.5%) and the thermal stress group displaying the lowest (32.78%) compared to the control. Similar to tenacity, the coefficient of variation for elongation measures the variability within each group. The Vitamin C supplemented group (T2) had the highest CV% for elongation, indicating relatively higher variability. Lee, (1999) records that the elongation of raw silk typically falls within the range of 18-23%, and considerable elongation can minimize winding breaks. The findings in the present study align with this observation. The positive correlation between vitamin C supplementation and increased tenacity suggests that vitamin C may enhance the overall strength of silk fibers. This finding aligns with the potential role of vitamin C in promoting the synthesis of collagen, a protein known for its structural strength (Chambial, *et al*., 2013). The observed variation in elongation values could be attributed to the complex interplay of factors influencing silk production, such as genetic characteristics, environmental conditions, and nutritional factors (Seem, 1922).

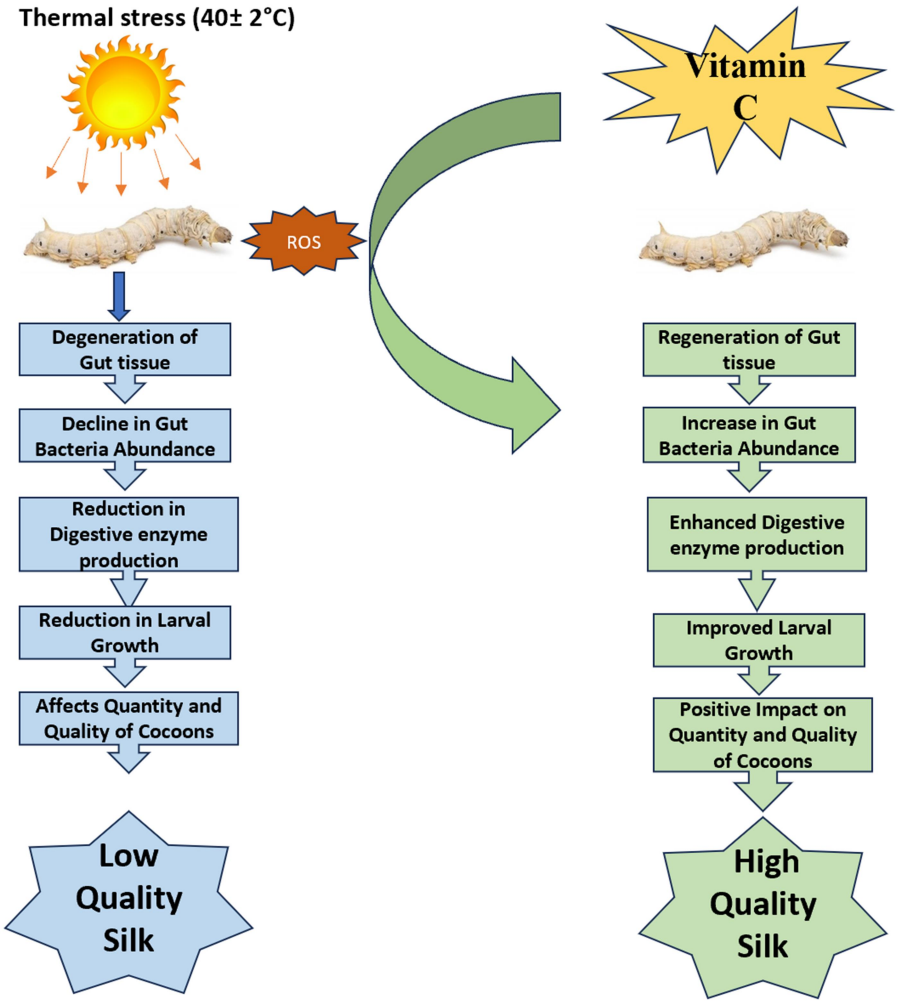
The observed results indicated that the group supplemented with vitamin C under thermal stress, exhibits an improvement in both tenacity and elongation when compared to the group subjected to heat shock alone. This suggests that the addition of vitamin C under thermal stress conditions may have a positive impact on the strength (tenacity) and stretchability (elongation) of the silk fibers, potentially mitigating the adverse effects of thermal stress on these parameters. The observed upregulation implies a beneficial role of vitamin C in enhancing the mechanical

properties of the silk, which could be significant for improving the overall quality and usability of the silk fibers.

Nutrition emerges as a critical factor influencing both larval and cocoon development. The improved quality of ascorbic acid-treated mulberry leaves contributes to increased yield and better cocoon quality. This finding resonates with the observations of Bose *et al.,* (1995), who reported that succulent mulberry leaves with less fiber and higher mineral content stimulate metabolic activities in silkworms, resulting in qualitative improvements in cocoons and silk. The observed consistently better rearing performance in the ascorbic acid-treated group may be attributed to the higher concentration of proteins and digestive enzymes resulting from improved gut bacterial composition. This suggests a potential link between ascorbic acid supplementation, gut health, and the subsequent economic traits of cocoon production. The observed improvements in economic parameters such as cocoon length, width, weight, shell weight, filament length, weight, size, and tensile strength suggest the potential for incorporating antioxidant supplements in sericulture practices.

In conclusion, our study adds a novel dimension to sericulture practices by exploring the positive impact of ascorbic acid supplementation on the economic traits of cocoon production under thermal stress. The multifaceted roles of ascorbic acid, encompassing antioxidant activity, gut health enhancement, and improved protein synthesis, position it as a promising tool for the development of resilient and high-yielding silkworm breeds. The findings open avenues for further research into the intricate interplay between nutritional supplements**,** environmental stressors, and the economic outcomes of sericulture.

Figure 5.1. Schematic overview of impact of supplementation of vitamin C on the gut of silkworm larvae on exposure to thermal stress



CHAPTER 6

CONCLUSION

6.Conclusion

- In conclusion, this study delved into the pivotal fifth instar stage of silkworms, recognizing its heightened metabolic and physiological activities. The gut, a linchpin in digestion and absorption, was chosen as the focal point, with gut bacteria proving indispensable for normal silkworm life functions. The research's overarching goal was to explore the potential of vitamin C supplementation in mitigating thermal stress challenges faced by silkworms, employing a comprehensive analysis spanning gut bacteria, protein and enzyme concentrations, gene expression, gut histology, and economic parameters.

- Thermal stress negatively affects larval growth, metabolism, gut bacterial concentrations, and overall cocoon quality. However, vitamin C supplementation demonstrates a remarkable ability to counteract these adverse effects. The study highlights the intricate dynamics between environmental stressors, nutritional supplementation, and gut microbial communities, emphasizing the crucial role of diverse gut microbiota in silkworm health and development.

- The examination of gut bacteria through culture method revealed dominant genera, including Bacillus, Lactobacillus, Citrobacter, Pseudomonas, Enterobacter, and Klebsiella. Bacillus sp. Was the common bacteria isolated from the four groups.

- Notably, the 16S rRNA metagenomic study unveiled variations in bacterial populations, showcasing decreased diversity in temperature-exposed groups and enhanced abundance in vitamin C supplemented groups. Vitamin C demonstrated a nuanced impact, reducing overall bacterial abundance but selectively enhancing beneficial species like Citrobacter, Klebsiella, Erwinia, Pseudomonas, Enterobacter, Clostridium, and Enterococcus. These bacteria are useful for the production of digestive enzymes.

- Moreover, the research illuminated vitamin C's role in influencing larval growth, particularly under temperature stress, where the supplemented group exhibited superior growth and yield attributes.

- Biochemical analyses highlighted the sensitivity of protein concentration and digestive enzyme activity to environmental stressors, showcasing decreases in the

thermal stress group and elevations in vitamin C supplemented and under thermal stress groups.

- Our study not only elucidates the impact of thermal stress and vitamin C supplementation on digestive enzyme activity but also provides valuable insights into the specific bacterial players involved in food degradation. The varying presence of Bacillus, Klebsiella, Citrobacter, and Pseudomonas in response to thermal stress and vitamin C supplementation underscores the intricate interplay between environmental factors, gut microbial communities, and enzymatic processes. This knowledge contributes to refining sericulture practices by offering a nuanced understanding of the factors influencing the digestion pathway in silkworm larvae, thereby paving the way for targeted interventions to optimize cocoon production and quality.

- mRNA expression of Hsp genes (Hsp 20.4, Hsp 40, Hsp 70 and Hsp 90) in silkworm gut indicated distinct responses, with heightened expression in the heat shock-exposed group, reduced expression in the vitamin C supplemented group, and the highest expression in the vitamin C supplemented under heat shock group.

- Economic parameters of cocoon production were scrutinized, revealing positive impacts of vitamin C supplementation under thermal stress on parameters such as cocoon weight, length, and width, shell weight and percentage, filament length, weight, size, and tensile strength of silk fiber.

- Our findings highlight the detrimental impact of thermal stress on various crucial aspects of silkworm development and cocoon production. Larval growth, metabolic activities, and gut bacterial concentrations were adversely affected, contributing to the production of economically subpar cocoons in the thermal stress-exposed group. These findings underscore the vulnerability of silkworms to environmental stressors and emphasize the importance of addressing these challenges for optimal sericulture outcomes.

- Conversely, the introduction of 0.2% vitamin C as a supplement exhibited a remarkable positive influence across the board. Larval growth and metabolism experienced notable elevations in groups treated with vitamin C, indicating the

beneficial impact of this supplement on the overall health and development of silkworms. Importantly, even under conditions of thermal stress, where the challenges were most pronounced, the group supplemented with both thermal stress and vitamin C demonstrated a remarkable resilience and improvement compared to the thermal stress-only group.

- These results strongly advocate for the inclusion of vitamin C supplementation in sericulture practices as a viable strategy to counteract the negative effects of thermal stress, ultimately leading to enhanced larval growth and the production of economically superior cocoons.

- In essence, this study unraveled interactions between environmental temperature, nutritional supplementation, and the dynamics of gut microbial communities, offering valuable insights for optimizing sericulture practices and contributing to broader understandings of insect physiology and applied entomology.

CHAPTER 7

RECOMMENDATIONS

7. Recommendations

These recommendations aim to guide upcoming scholars in addressing critical gaps in knowledge, refining practical applications, and contributing to the sustainability and resilience of sericulture practices.

- Exploring Mechanisms of Resilience: Future research can delve into the specific molecular and physiological mechanisms underlying the resilience observed in silkworms under thermal stress when supplemented with vitamin C. Investigating gene expression patterns, metabolic pathways, and signaling cascades could provide a more in-depth understanding of how vitamin C mitigates the negative effects of thermal stress.

- Microbiome Modulation Strategies: Given the significant impact of gut bacteria on silkworm health, researchers can explore strategies to modulate the silkworm microbiome for enhanced resilience. This could involve targeted interventions to promote the growth of beneficial bacteria or the use of probiotics to support gut health under stress conditions.

- Further exploration of specific bacterial species involved in digestion and absorption, understanding their responses to thermal stress and vitamin C supplementation, and optimizing food digestion in sericulture practices. This knowledge contributes to a broader understanding of insect-microbe interactions and their impact on digestive processes, paving the way for advancements in insect physiology and applied entomology.

- Long-Term Effects and Generational Impact: Investigating the potential long-term effects of vitamin C supplementation on silkworm populations could provide insights into generational impacts. Understanding whether the positive effects observed in larval stages persist through pupation and influence subsequent generations is essential for sustainable sericulture practices.

- Field Trials and Application in Sericulture Practices: Conducting field trials to validate the efficacy of vitamin C supplementation under real-world sericulture conditions is paramount. This could involve testing the supplementation strategy on a larger scale and assessing its impact on cocoon

production, silk quality, and overall economic parameters in practical sericulture settings.

- Environmental Factors and Interactive Effects: Exploring how other environmental factors, such as humidity, light, and food quality, interact with thermal stress and vitamin C supplementation is crucial. Understanding the holistic environmental context will contribute to developing comprehensive strategies for sericulture management.

- Comparative Studies with Other Stressors: Conducting comparative studies with other stressors commonly encountered in sericulture, such as pathogen exposure or fluctuating temperatures, can provide a broader perspective on the versatility and specificity of the observed responses to vitamin C supplementation.

- Integration of Advanced Technologies: Incorporating advanced technologies like omics approaches (genomics, transcriptomics, metabolomics) and imaging techniques can offer a more detailed and holistic view of the molecular and physiological changes occurring in silkworms in response to thermal stress and vitamin C supplementation.

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