Probiotics Supplementation to Mulberry Silkworm *B. mori*

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Abstract
This paper evaluates the importance, characteristics, mechanisms, and effects of probiotics added to silkworm *B. mori* feed. Scientific databases (PubMed, Science Direct, Google Scholar, MDPI, Elsevier) were screened. We are searching for alternative solutions to improve the nutritional quality of the mulberry leaves. Probiotics present properties such as non-pathogenetic or non-toxicity, adhere to epithelial cells, can be reproduced, stimulate immune response, have a positive influence on the host, can survive in intestinal mucosal surface, etc., and perform various functions that recommend them as natural feed supplements in silkworm nutrition. *Lactobacillus*, *Bifidobacterium*, *Bacillus*, *Streptococcus*, and *Saccharomyces* species are the most evaluated probiotics used in insects. In addition to producing lactic acid, probiotics also lower intestinal pH, inhibit pathogen populations, alleviate inflammation in the gut, boost immunity, and improve overall intestinal health. Silkworm gut contains various bacterial phylotypes (Proteobacteria, Firmicutes, Actinobacteria, and Bacteroidetes) that are crucial in nutrients metabolism and exhibit metabolites and enzymes indispensable for growth. This review highlighted that supplementing mulberry leaves with probiotics represents an eco-friendly strategy to improve the silkworm's performance, economic traits, and health.

Keywords: *B. mori*, gut microbiota, probiotics, performance, health.

1. Introduction

The *Bombyx mori* L. silkworm is an economically important lepidopteran insect for silk production due to its ability to convert protein from mulberry leaves into silk [1, 2]. It is known that silkworms, as a monophagous insects, require certain nutrients (proteins, amino acids, essential sugars, fatty acids, vitamins and micronutrients) for their growth and production that are provided by the fresh mulberry leaves (*Morus* spp.) as unique natural source of feed [3-5]. The quality of mulberry leaves depends on environmental conditions and affects the cocoons production and silk quality. Silkworm larvae's nutrition and health status are the main factors influencing silk production. Over time, researchers searched alternatives to enrich the mulberry leaves with nutrient supplements such as amino acids (glycine, asparagine, arginine, serine), vitamins (vitamin B-complex, vitamin C) and minerals (calcium, phosphorus, zinc, magnesium, potassium, copper, selenium) to improve its quality and to increase the silkworm productivity and health. [6].

This review highlighted the trends in silkworm nutrition by using microbial species *Lactobacillus*, *Bifidobacterium*, *Bacillus*, *Staphylococcus*, *Streptococcus* as natural ways to supplement the mulberry leaves and improve silkworms' larval and cocoon traits. Probiotics have been reported to produce lactic acid, but they also lower the pH of the intestinal tract, inhibit the growth of pathogens, reduce inflammation in the gut, increase immunity, and enhance the general health of the intestinal tract. The characteristics and modes of action of probiotics, the intestinal microbiota, and the functional role of gut bacteria in silkworms are also presented in this review.

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2. Materials and methods

Various scientific databases (PubMed, Science Direct, Google Scholar, MDPI, Elsevier) were screened to analyze and summarize the research on probiotics, their application and their effects on silkworm nutrition. Keywords used were "*B. mori* silkworm", "probiotic strains", "probiotic mechanisms", "microorganisms", "gut microbiota", "functional role of gut bacterial species", "nutrition", "mulberry leaves supplementation", "silkworm productivity and health".

3. Definition, properties, and mechanisms of action of probiotics

Every organism has a microbial ecosystem that might be beneficial to its health. The finding of probiotics made it possible to investigate the health effects of supplying helpful microbes to insects. Specifically, the microorganisms' impact is evaluated to enhance growth and reproductive efficiency and lower the incidence of illnesses under demanding conditions during raising [7].

Food and Agriculture Organization of the United Nations (FAO) and World Health Organization (WHO) [8], defined probiotics as "live microorganisms which, when administered in adequate amounts, confer a health benefit on the host". The selection of microorganisms as probiotics requires specific properties or characteristics (Table 1) related to their safety, performance, and technological aspects [9].

Table 1. Specific properties of probiotic strains[1]

Aspects	Probiotics strain properties
Safety	✓ Non-pathogenic, non-toxic, non-allergic, and non-mutagenic. ✓ Able to adhere and colonize the epithelial cell lining to establish itself in the colon. ✓ The ability to adhere to the epithelium secures the strain from being easily flushed out by peristaltic movements.
Performance	✓ Able to survive in sufficient numbers and adhere to the intestinal mucosal surface. ✓ Stimulate an immune response that positively affects the host. ✓ Survive the environmental conditions in their target site of action and proliferate in this location.
Technological	✓ Probiotic strains should be easy and inexpensive to reproduce. ✓ Large-scale production, good viability, and stability of the product during storage. ✓ Stress resistance during processing and storage, with the process and product application robustness. ✓ Able to survive particularly in the harsh environmental conditions of the small intestine (e.g., gastric and bile acids, and digestive enzymes). ✓ Resistant to phages and to have good sensory properties.

[1] Source: Priyadharshini et al. [9]

Table 2 summarizes the major mechanisms of action of probiotics: i). epithelial barrier improvement, ii). increased adhesion to the intestinal mucosa, iii). inhibition of pathogen adhesion, iv). competitive exclusion of pathogenic microorganisms, v). production of anti-microorganism substances, and vi). immune system modulation [10]. Probiotic microbial flora activity in the host's gut may improve feed absorption and digestion efficiency, boosting the host's production and assimilation of proteins [11]. Additionally, probiotics break down easily absorbed substances and can even generate specific vitamins, which may aid in nutritional absorption. According to the theory that beneficial microbial ecology is crucial for eukaryotic metabolism, insects require a variety of enzymes from their gut microbial flora to aid in the digestion of feed components. These enzymes can then help release other molecules, such as fermentable sugars and amino acids, that benefit the insect's growth [12].

Table 2. Mechanisms of action of probiotics[1]

Mechanisms		
Epithelial barrier improvement	✓	The mucous layer, antimicrobial peptides, secretory IgA, and the epithelial junction adhesion complex are the intestinal barrier's defences, which play a significant role in maintaining epithelial integrity and protecting the organism.
Increase adhesion to the intestinal mucosa.	✓	Colonization of the intestinal mucosa by probiotic bacteria depends on their adhesion to it. Their surface adhesions facilitate the interaction of lactic acid bacteria with intestinal epithelial cells and mucus.
Inhibition of pathogen adhesion	✓	Mucin, the main component of mucus, is a complex combination of glycoproteins generated by the host intestinal epithelial cells that inhibits the attachment of harmful microorganisms.
Competitive exclusion of pathogenic microorganisms	✓	A probiotic's effectiveness may be assessed by its capacity to adhere to gut receptor sites. The adherence of harmful bacteria is inhibited, and the binding of probiotic bacteria prevents their colonization. By producing a hostile microecology, removing available bacterial receptor sites, secreting antimicrobial compounds and selective metabolites, and competitively depleting vital nutrients, probiotic bacteria reduce the growth of pathogens or prevent them from colonizing. The process by which bacteria compete with one another for available nutrients and mucosal adhesion sites underlies the competitive exclusion of harmful species. For example, bacteria can produce antimicrobial compounds like lactic and acetic acid to alter their environment and make it less favourable for other bacteria.
Production of anti-microorganism substances	✓	Short-chain fatty acids, which are produced by probiotics, reduce intracellular pH and prevent the development of pathogenic microorganisms. Bacteriocins and antimicrobial peptides are two examples of the antimicrobials released by several probiotic organisms. The active spectrum of bacteriocins, such as lactacin, plantaricin, and nisin, against some pathogens is limited.
Modulation of the immune system	✓	By interacting with epithelial and dendritic cells, as well as with monocytes/macrophages and lymphocytes, probiotic bacteria may possess an immunomodulatory influence on the host.

[1]Source: Bermudez-Brito et al. [10]

4. Microorganisms used as probiotics in silkworm

For many insect species, microorganisms are crucial to their growth and development. The microbial species *Lactobacillus*, *Bifidobacterium, Bacillus, Streptococcus*, and *Saccharomyces* are the most often evaluated probiotics used for insects [13]. These microbes are between the seven "core" microorganisms most frequently utilized as probiotics by the World Gastroenterology Organization (WGO). Since *Enterococcus* is a frequent symbiont in the intestinal tract of insects, particularly in Lepidoptera, the group is significantly neglected. The prevalence of both pathogenic and probiotic strains of *Enterococcus* might explain this [14]. In addition to producing lactic acid, probiotics also lower intestinal pH, reduce pathogen populations, alleviate inflammation in the gut, boost immunity, and improve overall intestinal health [15].

Lactobacillus spp. is lactic acid–producing Gram-positive, non-sporulating, and anaerobic bacteria that benefit gut health [16]. *Bifidobacterium* spp. is an anaerobic, Gram-positive, non-sporulated, pleomorphic bacterium that produces lactic and acetic acids as by-products of glucose metabolism [17]. *Bacillus* spp. are Gram-positive, spore-forming microorganisms with probiotic effects on the host by producing lactic acid. In contrast, some other species of *Bacillus* may produce toxins in the host organism [18]. *Saccharomyces* spp. refers to the non-pathogenic yeast bacterium *S. cerevisiae*, which is usually used as a probiotic for its immunomodulatory effects [19]. Probiotic effects are known to be strain-specific, thus claims about their possible health benefits should be avoided. As a result, even within the same species, a strain's health benefits may not transfer to another strain [10].

5. Intestinal microbiota of *B. mori* silkworm

Several different bacteria are found in the midgut of the *B. mori* silkworm, which helps in metabolic activities. However, little is known about the diversities and characterization of these bacteria [20] and how they contribute to the silkworm's growth, development and health [1].

The study of Kalpana et al. [21] investigated the gut microbiota throughout the *B. mori* life cycle and reported higher intestinal bacterial microflora in the last larval instars (fourth and fifth instar), correlated to the active feeding stage to improve nutrient digestion and growth. Hui et al. [22] used the PCR/DGGE approach and 16S rDNA gene library analysis to identify 41 bacterial phylotypes found in the midguts of *B. mori* silkworm larvae. Similarly, using the 16S rRNA technique, Subramanian et al. [23] demonstrated the presence of *Bacillus subtilis*, *Pseudomonas fluorescens*, and *Streptomyces noursei* in the silkworm intestinal microflora. Prem Anand et al. [24] reported that *B. mori* gut is colonized from the fifth instar by 11 bacterial phylotypes associated with polysaccharide degradation that impacts nutrient absorption, digestion and insect development. Cellulolytic bacteria that improve nutrient metabolism and growth have also been found in silkworm gut microflora by Khyade and Marathe [25]. Li et al. [26] analysed the composition of gut bacteria in two silkworm strains by 16S rRNA gene sequencing and observed significant differences in their gut bacterial profiles. Yeruva et al. [1] used a metagenomic technique to identify the potential probiotic bacterial populations from the gut of silkworm *B. mori*, reported as the predominant genus *Lactobacillus* (*L. plantarum*, *L. rhamnosus*, *L. paracasei* and *L. acidophilus*), followed by *Enterococcus* and *Bacillus*. The authors recommended that *Lactobacillus* and *Bacillus* spp. could be used as a feed supplement to mulberry leaves to boost the economic traits of silkworms. The probiotic potential of indigenous lactic acid bacteria from intestinal *B. mori* was also noticed by Saranya et al [27].

Moreover, numerous studies demonstrated the beneficial effects of silkworm microbiota against infectious diseases [28-30]. Recently, Dee Tan and Bautista [31] investigated the bacterial diversity of four Philippines silkworm strains using 16S rRNA gene amplicon sequencing analysis and found the abundance of five bacterial genera (*Pseudomonas*, *Sphingomonas*, *Delftia*, *Methylobacterium*, and *Acinetobacter*) similar to that of other silkworm strains.

The gut bacteriome has received most of the attention in studies on the microflora of silkworms, while the fungal gut communities are primarily unknown. According to Chen et al. [32], Mwchahary and Brahma [33], there were bacteria from the phyla Proteobacteria, Firmicutes, Actinobacteria, and Bacteroidetes present together with fungi from the phyla Ascomycota and Basidiomycota. Studies have shown that the yeast microbiota of *B. mori* gut contain *Cryptococcus rajasthanensis* and *Blastobotrys bombycis* sp. [34-36].

However, several factors, including the host's genotype, nutrition, age, and environment, can influence the composition and quantity of gut bacteria in silkworms [33].

6. Functional role of gut bacteria in silkworm

Gut microbiota is a crucial regulatory component for gut health [37] due to their beneficial symbiont characteristics and role [38]. Their metabolic essential enzymes are needed to break down the substrates and release nutrient-critical molecules [39]. The silkworm gut bacterial communities have also been evaluated for the production of some digestive enzymes needed to digest leaf nutrients [40]. Several studies reported the vital functional roles of silkworm gut microbiota related to nutrient digestion, absorption, and detoxification, innate immunity enhancement, supplying nutrients and antibacterials, growth-promoting metabolites, and management of diseases [36, 41-43]. Table 3 presents the functional role of gut bacteria species in the *B. mori* silkworm host.

Table 3. Role of gut bacteria species in *B. mori* silkworm host

Species	Functions	Reference
Lactobacillus	✓ Stimulated growth factors and improved economic characteristics.	Yeruva et al. [1]; Sing et al. [44]; Suraporn et al. [45]; Suraporn and Terenius [46]
Bifidobacterium	✓ Immunomodulatory effect by increasing the activity of protease, amylase and invertase; improve production of raw silk.	Taha et al. [40]
Bacillus subtilis	✓ Release secondary metabolites, vitamin B synthesis, produce antimicrobial peptides (AMP), and increase resistance.	Li et al. [47]
Bacillus aryabhattai and *Bacillus* sp.	✓ Cellulolytic activity.	Pandiarajan and Revathy [48]
Bacillus megaterium	✓ Glycolysis of starch.	Prasanna et al. [49]
Bacillus pumilus	✓ Digestive system and protection against antiviral agents nuclear polyhedrosis virus (BmNPV).	Liu et al. [42]
Bacillus licheniformis	✓ Production of extracellular enzymes.	Mala and Vijila [50]
Bacillus sp., Brevibacterium sp., Corynebacterium sp., Staphylococcus sp., Klebsiella sp., and *Stenotrophomonas sp.*	✓ Digestion and synthesis of lipase enzyme.	Feng et al. [51]
Enterococcus sp. and *Staphylococcus sp.*	✓ Mechanism of defence against infection.	Sun et al. [52]
Enterococcus mundtii	✓ Host metabolism (production of metabolites and lactic acid).	Liang et al. [43]
Enterococcus casseliflavus	✓ Growth and development through the synthesis of L-tryptophan.	Liang et al. [53]
Enterococcus faecalis	✓ Immunity against infection.	Zhang et al. [54]
Staphylococcus gallinarum	✓ Defence mechanism.	Gibson et al. [55]
Staphylococcus gallinarum SWGB 7 and *Staphylococcus arlettae SWGB 16*	✓ Stimulated growth, improved economic traits and immune system.	Saranya et al. [27]
Streptomyces noursei	✓ Antimicrobial activity in relation to disease control.	Subramanian [23]; Mohanraj et al. [56]
Enterobacter aerogenes, pneumoniae sp. Pneumonia, Yersinia enterocolitica	✓ Growth and development, host resistance, chemical pesticide degradation, and entomopathogen host antagonism interactions.	Ramesh et al. [57]

7. Effects of probiotics supplementation of mulberry leaves in silkworm

Table 4 summarizes the literature studies that used probiotic supplementation of mulberry leaves in silkworms, with special emphases on *Lactobacillus* and *Bacillus* spp., and the main results on productivity and health status.

Table 4. Effects of probiotics supplementation of mulberry leaves in silkworm

Probiotic	Dose/ Concentration	Instar	Main results	Reference
L. plantarum	ns	ns	↑ larval body weight, cocoon, shell weight and pupation rate.	Sing et al. [44]
L. acidophilus	10^8 cfu/ml	3 to 5th larval instars	↑ survival ratio, mature larval weight, pupation ratio, cocoon weight and cocoon-shell ratio. ↑ silk yield and silk harvest.	Suraporn et al. [45]
L. casei	10^8 cfu/ml	5th instar	↑ larval weight, cocooning and pupation ratio, and economic characters (cocoon weight and size) of larvae infected with microsporidium *Nosema bombycis*. ↑ feed digestion and nutrient absorption.	Suraporn et al. [46]
L. rhamnosus *B. bifidum* and their combination	3 and 6%	4 to 5th larval instars	*L. rhamnosus* + *B. bifidum* ↑ larval performance and tissue growth, ↑ feed intake and assimilation, followed by *L. rhamnosus* → *Bifidum.* Both concentrations ↑ economic and silk (length, weight and size) parameters with no significant differences between them. Lower concentrations (3%) are recommended.	Moustafa and Soliman [58]
Spirulina, *S. cerevisiae,* *L. acidophilus* and *L. sporogens*	300 ppm of each	ns	Spirulina and yeast ↑ cocoon characteristics, fibroin content and silk quality, followed by *L. acidophilus* and *L. sporogens*.	Masthan et al. [59]
B. cereus, *B. subtilis,* *B. amyloliquefaciens,* *L. casei,* *L. plantarum* and their combination	ns	3 to 5th larval instars	*B. amyloliquefaciens* ↑ silk gland proteins followed by combined bacterial diet → *L. casei* → *B. cereus* → *L. plantarum* → *B. subtilis.* *B. amyloliquefaciens* ↑ silk gland carbohydrates followed by *B. cereus* → combined bacterial diet → *B. subtilis* → *L. plantarum.* *B. amyloliquefaciens* ↑ silk gland lipids followed by *B. cereus* → combined bacterial diet → *L. casei* → *L. plantarum* → *B. subtilis.* *B. amyloliquefaciens* ↑ fibroin content followed by *B. cereus* = combined bacterial diet → *L. plantarum* → *L. casei* → *B. subtilis.* *B. amyloliquefaciens* ↑ digestion and nutrient absorption, ↑ protein synthesis and deposition in the body, ↑ silk gland and cocoons. *L. casei* ↑ length and weight of larval body and ↑ cocoon weight. *L. casei* ↑ protects against harmful bacteria and promotes growth rather than immune response. This study demonstrates the possible probiotic-boosting effect of *B. amyloliquefaciens, L. casei, B. cereus,* and a microbial community on silkworm physiology, metamorphosis, and silk production.	Sekar et al. [60]
B. licheniformis BMGB42 *B. niabensis BMGB17* and their mixture	10^4 cfu/ml 10^6 cfu/ml 10^8 cfu/ml	5th instar	*B. licheniformis* followed by *B. licheniformis* + *B. niabensis* (10^6 cfu/ml) ↑ larval weight, effective rate rearing, cocoon weight, shell weight, pupal weight, shell ratio, silk productivity and filament length; ↓ finer denier; ↓ larval mortality due to disease incidence.	Mala and Vijila [50]
Lact-Act (commercial probiotic based on *L. sporogens*, *B. thuringiensis*, yeast hydrolysate, a-amylase, vitamin-mineral mix)			↑ larval survival when exposed to bacterial pathogens (*B. thuringiensis* var. sotto. and *S. aureus*).	Rajakumari et al. [61]

8. Conclusions

Enhancing the quality of mulberry leaves is crucial for improving the appetite and/or feed quality, as it is one of the primary factors influencing the silkworms development in larval instars. Findings from the literature indicate that probiotics supplementation to mulberry leaves is a sustainable way to boost silkworm health, productivity, and quality of silk.

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