Effect of *Rhodotorula glutinis* yeast x mulberry leaves on morpho-productive characteristics of IBA polyvoltine breed of silkworm *Bombyx mori* L.

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Abstract

The mulberry leaves are the sole feed for the silkworm *Bombyx mori* L. The use of yeast has a great impact in the development of the morpho-productive parameters as shown in other studies and the results may differ by silkworm breed and the day of administration of the yeast. The objective of this study consists in evaluating the IBA silkworm performance, a polyvoltine breed, fed with mulberry leaves +/- *Rodotorula glutinis* yeast. The morpho-productive performances were determined during the trial in the 5th instar at 1st, 5th, 7th and 9th days. A total of 300 larvae were distributed randomly in 3 groups (100 larvae/group, 2 replicates each one): 1. C group fed mulberry leaves; 2. E1 fed C diet and yeast (1x10^{7}); 3. E2 fed C and yeast (1x10^{9}). Mulberry leaves treated with *R. glutinis* yeast, irrespective of concentration and days of measurements, had a positive influence on the morpho-productive parameters of IBA breed larvae (weight and average daily gain). The silk gland weight and cocoons traits (weight of the pupae, weight of raw cocoon and longitudinal and transversal axes) were superior in experimental diets.

Keywords: diet, IBA, mulberry leaves, *Rhodotorula glutinis*, silkworms.

1. Introduction

Mulberry leaves are the single diet of the silkworm *Bombyx mori L.* The leaves are rich in water, proteins, cellulose, mineral and vitamins, the content depending of many factors like: timetable for leaf picking, age of the leaf, type of the mulberry tree, degree of soil fertilization, weather, plantation type, cultural hygiene of the plantation, silkworm growth cycles.

Given the decline in silkworm breeding activity and the progressively smaller area used for mulberry cultivation, and for increasing the productivity of the sector as well, it was necessary to identify solutions to improve the quality of the mulberry leaf.

Over the years, scientists were able to find different ways to enrich the diet of silkworms through a diversity of methods. Adding amino acids and antibiotics were a success for illness resistance and molecular protein study in silkworms [1]. For improving the silkworm performance, supplements with vitamins such as folic acid, ascorbic acid, thiamin, niacin and multivitamins were studied by Joyce and Sabura in 2021 [2]. In other studies, were introduced probiotics such as *Saccharomyces cerevisiae* in combination with mulberry leaves for improving the economic parameters of *B. mori* larvae in their 5th instar [3]. The studies showed that the treatment with *S. cerevisiae* ameliorate immunomodulation properties and the growth performance [3]. Furthermore, an improvement of filament length production and spinning of the silk was registered [4].

In 2017 a trial was conducted aiming to protect *B. mori* from microbial disease attacks and to boost

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cocoons production by using leaves supplements with *Bifidobacterium bifidum* and *S. cerevisiae* [5]. The increase of cocoon production, silk quality and filament length continued to be the primary preoccupation of experts in the sector. Of a high interest remains producing a high-quality silk obtained from silkworms with improved morpho-productive parameters.

One type of yeast that is less known for its use in insects is *Rhodotorula glutinis*. Some species of *R. glutinis* are the main carotenoid-producing microorganisms with predominant synthesis of β-carotene, torulene and torularhodin [6]. Moreover, they are widely known as a good source of proteins, lipids and vitamins and may produce a wide range of metabolites, including carotenoids, and enzymes [7].

In 2024, a study was conducted by Hăbeanu et al. (*unpublished data*) using the *R. glutinis* yeast to fortify the nutritional qualities of mulberry leaves used for feeding two breeds of silkworm *B. mori* (RG-90 which belong to monovoltine type, and Maritza III, bivoltine type). The study demonstrated that the mulberry leaves supplemented with yeast allow to silkworms to express and ameliorate their morpho-productive potential (larva, silk gland, and cocoon characteristics), whatever the breed. According to that research, mulberry leaves treated with *R. glutinis* yeast for silkworm larvae feeding, significantly outperformed the Control group in terms of both morphological and productive features.

Based on voltinism, the *B. mori* has 3 types: monovoltine, bivoltine and polyvoltine. It was shown that the polyvoltine type allows the rearing of 5-6 larvae generations per year and are characteristic for tropical climates [8].

Polyvoltine larvae type has high viability, but technological qualities are lower [9]. The main weakness of polyvoltine silk is its poor fibre quality [10].

The objective of this study was to evaluate the impact of supplementing mulberry leaves with *R. glutinis* yeast with different concentration, on morpho-productive parameters of IBA polyvoltine silkworm breed and time influence on the larvae parameters.

2. Materials and methods

Biological material

The IBA breed is originally from India, discovered in 1980, and is characterized by grey embryony eggs, white larvae, and white cocoons with sharp ends. The IBA breed are conserved in the gene pool of the Research Station for Sericulture Baneasa Bucharest, Romania.

The *R. glutinis* yeast was provided from Bratislava and was conserved in the Intern Collection of IBNA Balotesti-Romania, within the Laboratory of Animal Nutrition and Biotechnology [4].

Study location

The experiment took place at Research Station for Sericulture Baneasa Bucharest (Romania), in June-July 2024, in standard condition and facilities for silkworm rearing.

Experimental design

The trial was conducted on 300 larvae *B. mori* belonging to IBA breed in 5th instar that were randomly distributed into 3 groups (100 larvae per group) with 2 replicates each one (Table 1): 1) Control (C) fed with mulberry leaves; 2) Experimental 1 (E1) fed C diet plus *R. glutinis* yeast in concentration 1x10^7; 3) Experimental 2 (E2) fed C diet plus *R. glutinis* yeast in concentration 1x10^9.

Table 1. Experimental design

Silkworm breed	Group	Treatment
IBA, 5th instar larvae	C	Mulberry leaves
	E1	Mulberry leaves + *R. glutinis* yeast in concentration 1x10^7
	E2	Mulberry leaves + *R. glutinis* yeast in concentration 1x10^9

C= Control; E1 = Experimental group 1; E2 = Experimental group 2.

Preparation of yeast solution

The yeast was activated for 24-48 h at 28 °C, 150 rpm under aerobic conditions and sub-cultured at least three times in yeast-peptone-dextrose (YPD) broth medium (Himedia, M1365). The following components were present in the YPD broth in a 1:10 (w/v) ratio: 1% yeast extract, 2% dextrose mixed in distilled water, and 2% peptic digest of animal tissue. pH was adjusted to 6.5±0.2 before sterilization at 121°C for 15 minutes.

The solution was prepared as follow:
- $1x10^7$ yeast colony forming units per ml combined with 1L distilled water for E1 group;
- $1x10^9$ yeast colony forming units per ml combined with 1L distilled water for E2 group.
The resulted solution was held in the laboratory fridge.

Diets
During the 5th instar period (9 days), first meal of the day was composed by 100 g of mulberry leaves for the C group, and for the E1 and E2 groups, mulberry leaves 100 g for each group were sprayed with 40 ml of *R. glutinis* solution with known concentration, $1x10^7$ or $1x10^9$ yeast solutions, which was used in silkworm fed. The rest of the meals were administrated only mulberry sprouts.

Measurements
At 1st day (D), 5th D, 7th D and 9th D of the 5th instar from each group were randomly extracted 10 larvae for weight (g) and length (mm) measurements. The weight of silk gland was determined at 5th D, 7th D and 9th D of the 5th instar from 4 larvae per group. After cocoons spinning, 10 raw cocoons per group were randomly selected for the cocoon weight, shell weight, pupae weight measurements and the shell/weight ratio were calculated. An electronic scale and digital caliper were utilized for this.

Chemical analyses
The gross chemical composition of the mulberry leaves evaluated by methods approved by Commission Regulation (EC) no. 152 (OJEU, 2009).

Statistical analyses
For statistical interpretation of the experimental data was used Statistical Package SPSS software, version 20 (2011), General Linear Model (GLM) multivariate test. The Least Significant Difference (LSD) was used to compare mean differences.
The dependent variables were considered morpho-productive parameters and the fixed factor was diet. The Pearson correlation was applied in order to evaluate the relationship between parameters. Variations were considered significantly when P values $P \leq 0.05$ or highly significantly when P values < 0.01; 0.001, or 0.0001, and tendency was considered at $P<0.10$.

3. Results and discussion

This study shows the impact of fortifying mulberry leaves with *R. glutinis* yeast.
Several studies investigated the impact of application of various probiotics on mulberry leaf for improving silk quality and quantity and health status of silkworm. Previous studies reported some promising results on cocoon quantity parameters by using certain microorganisms such as *Spirulina* and *S. cerevisiae* [11].
The quality of mulberry leaves for feeding silkworms is a key factor of the success of cocoon production [12]. At the beginning of the trial, chemical analyse of the diets was done (Table 2). By comparing E1 and E2 diets with C we noticed that *R. glutinis* improved certain nutritional substances (fat and fibre).

Table 2. Chemical composition of the mulberry leaves (% DM)

Group*	Dry matter (%)	Protein	Ether extract	Crude fibre	Ash
C	25.44	25.79	1.79	13.67	12.34
E1	25.29	24.59	2.51	15.24	11.24
E2	28.33	25.10	2.49	15.41	13.61

*C = Control; E1 = Experimental group 1; E2 = Experimental group 2

Our results showed that by supplementing the mulberry leaves with different amounts of *R. glutinis* yeast solution (E1 and E2) the silkworms' performances were favourably modified (Table 3). Larval characteristics are very important for the development and overall productivity of the silkworms. The cocoon parameters directly impact the quality and quantity of silk produced [13].
Effect of *R. glutinis*, as supplement in silkworm feeding, has positive impact over the larval development parameters of polyvoltine breed IBA, no matter of concentrated solution but, major effect was seen at a concentration of $1x10^9$ yeast solution. The E2 group had 13% more final weight gain compared with C ($P=0.03$) The treatment tends to increase the final weight in E1 group compared to C group ($P= 0.09$). Average daily gain was significantly influenced in both experimental groups, with more pronounced impact in E2 fed group (16% higher in E2 *vs.* C group, respective 8.5% higher in E1 group, $P= 0.05$). Length of larva was positively impacted by the yeast addition as

well. The differences occurred in both experimental groups. In E1 group (P= 0.038) larvae had 3.96% higher length than C while in E2(P= 0.031) group the length was 4.13% greater compared with C. Between E1 and E2 mean values of larval length were not significantly influenced.

Table 3. Effect of mulberry leaves supplemented with *R. glutinis* on larval development

Parameters	Group			SEM	P-value
	C	E1	E2		
Initial weight D1 (g)	0.525	0.545	0.546	0.007	0.364
Final weight D9 (g)	2.644^{Tb}	2.839^{T}	3.00^{a}	0.049	0.010
Average daily gain (g)	0.235^{b}	0.255^{a}	0.273^{a}	0.005	0.020
Initial length D1 (mm)	33.280	34.336	33.926	0.227	0.160
Final length D9 (mm)	60.953^{b}	63.371^{a}	63.472^{a}	0.481	0.051

Abbreviations: C = Control; E1 = Experimental group 1; E2 = Experimental group 2; D = day; SEM = standard error mean. ^{a,b} Mean values with different superscript in the row indicate differences between dietary treatments (P< 0.01, highly significant; P≤0.05, significant; P<0.10, tendency (T) to be influenced by treatments).

In Table 4 are presented correlations between growth parameters. The results shown that final weight and average daily gain were strongly correlated (R=0.991; P<0.0001), and final weight and final length were moderately correlated (R=0.668; P<0.0001).

Table 4. Pearson correlation between final larval growth parameters

Correlations		Average daily gain (g)	Final length D9 (mm)
Final weight D9 (g)	R	0.991	0.668
	P*	0.0001	0.0001

*P<0.0001, highly significant correlations.

Figure 1 and 2 represents growth and length evolution during 1st, 5th, 7th and 9th D.

Mean values evolution for larval weight was:

- C group: D5 had a growth of 3.2 times higher compared to D1, D7 *vs.* D1 was 4.78 higher and D9 *vs.* D1 was 5.04 times higher.
- E1 group: D5 had a growth of 3.64 times higher compared to D1, D7 *vs.* D1 was 5.07 higher and D9 *vs.* D1 was 5.21 times higher.
- E2 group: D5 had a growth of 3.51 times higher compared to D1, D7 *vs.* D1 was 4.83 higher and D9 *vs*. D1 was 5.50 times higher.

Such as, in E2 fed group the growth evolution was predominant.

The pairwise comparison shows that larval growth evolution per day, and the comparison between days, results a highly significant difference (P < 0.001).

Mean larval length growth during the 1st, 5th, 7^{th,} and 9th D when measures were made per group showed us:

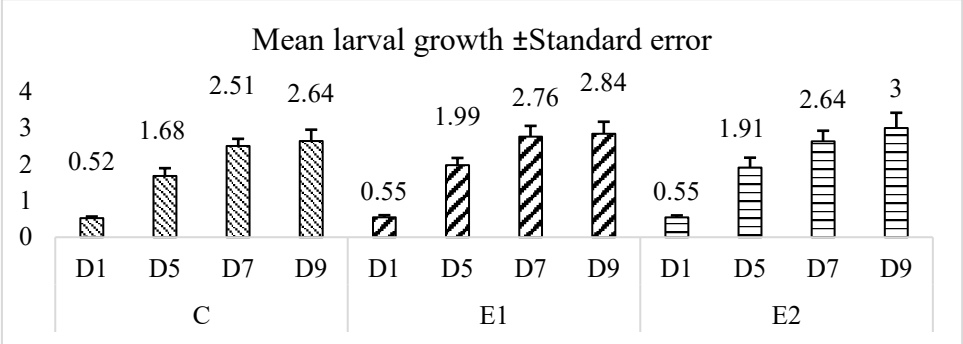
- C group: D5 had a growth of 1.49 times higher compared to D1, D7 *vs.* D1 was 1.77 higher and D9 *vs*. D1 1.83 times higher.
- E1 group: D5 had a growth of 1.55 times higher compared to D1, D7 *vs.* D1 was 1.74 higher and D9 *vs.* D1 1.85 times higher.
- E2 group: D5 had a growth of 1.53 times higher compared to D1, D7 *vs*. D1 was 1.81 higher D9 *vs.* D1 1.87 times higher.

As for growth, the length in E2 group had an overall higher increase than C group.

Length evolution per day, and differences of lengths measured in different days shows by comparison a highly significant evolution (P< 0.001).

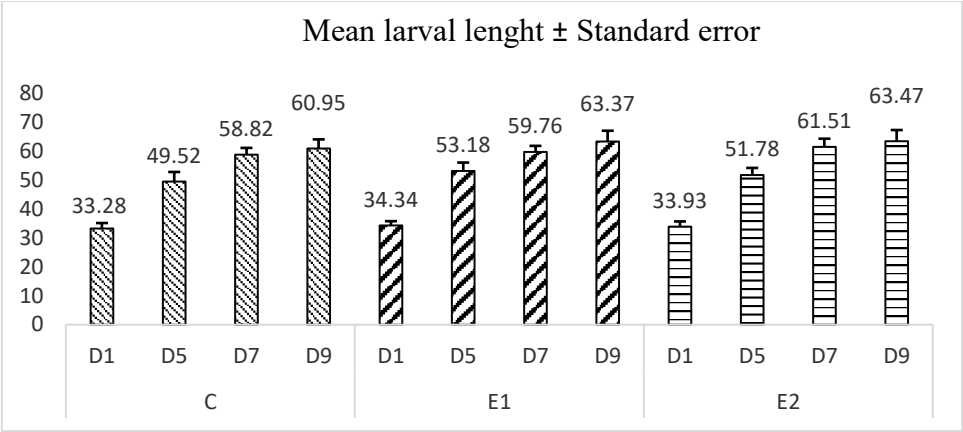
Previous study fed mulberry leaves supplemented with vitamin C during their 4th and 5th larval instars showed a significant increase in their length, whereas mature larvae fed on vitamin C showed a significant increase in their weight [14].

The silk gland of *B. mori* is a distinctive organ that synthesizes and secretes the proteins fibroin and sericin, two major components of cocoon silk [15]. Evolution of silk gland was another important factor to evaluate.



C = Control; E1 = Experimental group 1; E2 = Experimental group 2; D = day.

Figure 1. Larval weight (g) evolution as an effect of mulberry leaves supplemented with *R. glutinis* in 5th instar



C = Control; E1 = Experimental group 1; E2 = Experimental group 2; D = day.

Figure 2. Larval length (mm) evolution as an effect of mulberry leaves supplemented with *R. glutinis* in 5th instar

Pearson correlation (Table 5) between silk gland with larval growth and larval length reveals a moderate correlation between them. The silk gland was positive correlated (P<0.001) with productive parameters.

In Table 6, are presented diets and time influence on silk gland development as effect of *R. glutinis* yeast addition in silkworm feed.

Table 5. Pearson correlation between silk gland with larval growth and larval length

Pearson correlations		Larval growth (g)	Larval length (mm)
Silk gland (g)	R	0.520	0.497
	P*	0.001	0.002

*P< 0.001, highly significant; P≤0.05, significant correlation.

Table 6. Effect of mulberry leaves supplemented with *R. glutinis* on silk gland

Parameters	Group			SEM	P-value*
	C	E1	E2		
Silk gland D5 (mm)	0.233	0.238	0.195	0.012	0.342
Silk gland D7 (mm)	0.353	0.395^{T}	0.410^{T}	0.029	0.182
Silk gland D9 (mm)	0.560^{b}	0.710^{aT}	0.670^{T}	0.042	0.032

C = Control; E1 = Experimental group 1; E2 = Experimental group 2; D = day; SEM = standard error mean.
[a,b] Mean values with different superscript in the row indicate differences between dietary treatments (P<0.01, highly significant), tendency (T) to be influenced by treatments

For the C group, results revealed that in D9 silk gland was 2.4 times greater than D5. In comparison, E1 group had a growth of 2.98 times higher in D9 *vs*. D5. Meanwhile, a higher

pronounced effect was observed in E2 group, the length being in D9 of 3.44 times higher compared to D5. Feeding of silkworm larvae during the 5th instar with *R. glutinis* supplement influenced highly significant the silk gland traits. Nicula [16] used yeast hydrolysate solutions in silkworms and reported that they induced an increase in larval traits and silk gland weight without affecting the silk gland protein content.

As noted in Table 6, silk gland in D7, by comparison E1 *vs.* E2 had a tendency of growth (P=0.077) and in D9, C *vs.* E1 was considered significant higher (P= 0.012) and in the same time E1 vs. E2 had just a tendency for higher growth (P=0.063).

The raw cocoon characteristics (cocoon weight, shell weight, pupae weight and the ratio between weight of cocoon shell and weight of cocoon x 100) are presented in Table 7.

In term of cocoon weight E1 *vs*. C had a significantly higher increase (P=0.003), while in E2 group cocoon weight tend to be higher than C group (P=0.112). With regard to shell weight the mean values were similar between group (P>0.05).

Pupae weight was significantly higher in E1 compared to C (P=0.045) and C *vs.* E2 had a tendency (P=0.094), otherwise there is no significant effects. Ratio between weight of cocoon shell and weight of cocoon was not significantly different.

Pearson correlation (Table 8) shows a significant moderate influence between cocoon characteristics: raw cocoon weight with pupae weight (R=0.431; P=0.018) and shell weight (R=0.498; P=0.007). This study comes in support of Hăbeanu study on bivoltine breeds of silkworm (unpublished) that demonstrated that the mulberry leaves supplemented with yeast allow to silkworms to express and ameliorate their morpho-productive potential (larva, silk gland, and cocoon characteristics), whatever the breed.

Table 7. Effect of mulberry leaves supplemented with *R. glutinis* on raw cocoon characteristics

Parameters	Group			SEM	P-value
	C	E1	E2		
Raw cocoon weight (g)	1.225^{b}	1.445^{a}	1.390^{a}	0.042	0.087
Shell weight (g)	0.222	0.241	0.225	0.006	0.388
Pupae weight (g)	1.0^{bT}	1.199^{a}	1.164^{aT}	0.040	0.099
Ratio SW: CW (%)	18.303	17.054	16.371	0.499	0.286

C = Control; E1 = Experimental group 1; E2 = Experimental group 2; SW = Shell weight, CW = Raw cocoon weight; SEM = standard error mean. A tendency was considered at P<0.10.

Table 8. Pearson correlation between cocoon characteristics

Pearson correlations		Pupae weight (g)	Shell weight (g)
Raw cocoon weight (g)	R	0.431	0.498
	P*	0.018	0.007

*P≤0.05, significant correlation.

4. Conclusions

Amelioration of mulberry leaves nutritional quality by using *R. glutinis* yeast solution and feeding to the silkworms is a useful way to increase production values. The yeast manages to fortify silkworm larval growth, silk gland had a higher development which translates in more silk fiber and raw cocoon characteristics had a tendency for higher values.

Use of *R. glutinis* could solve multiple problems, especially in the current context of climate change. With a smaller mulberry plantation area in Romania and a lack of nutrients in mulberry leaves due to soil fatigue, there is a need to find optimal solutions to propagate the national heritage of silkworm breeds.

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