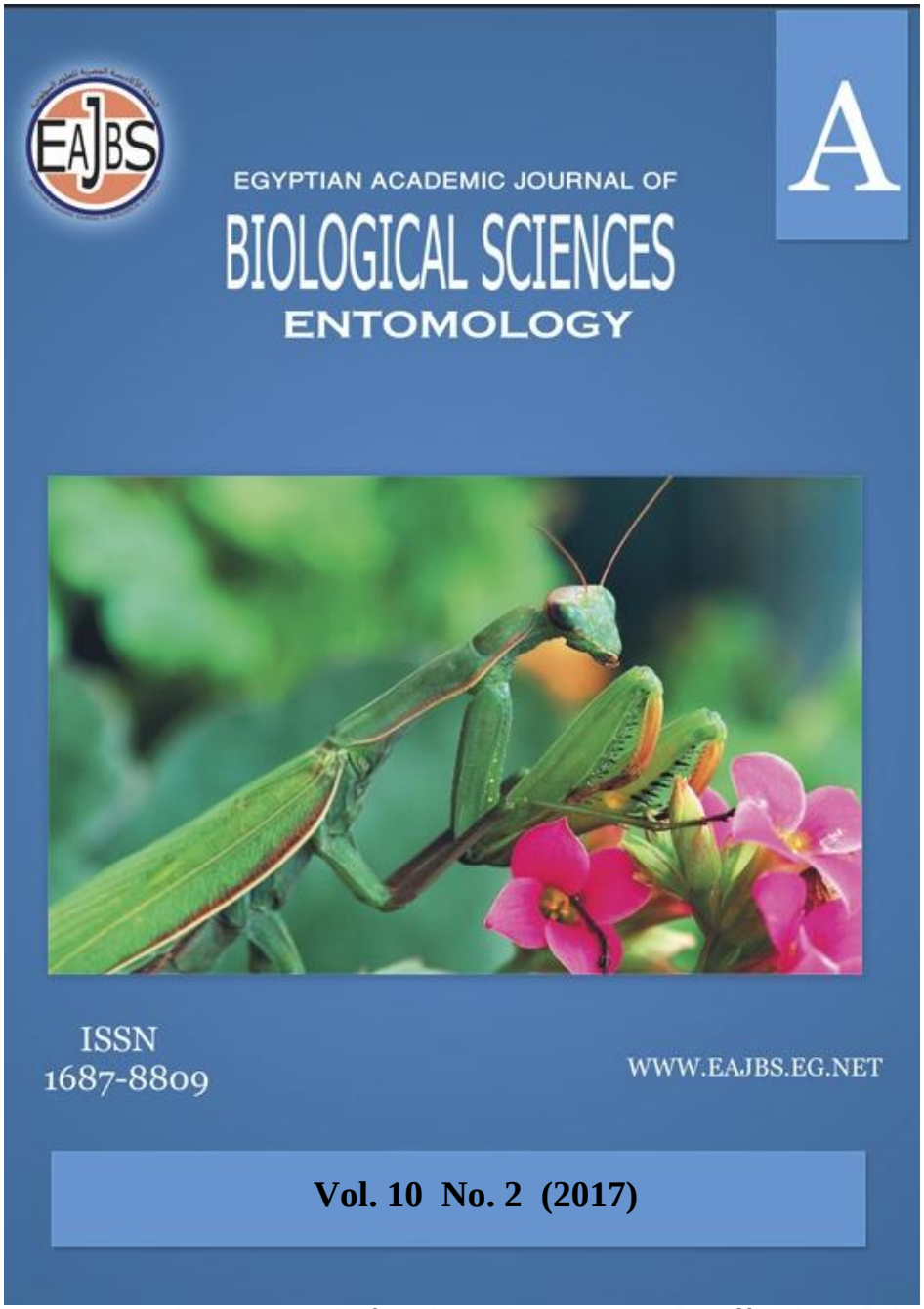

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Micro-Organisms Supplementation to Mulberry Silkworm, *Bombyx mori* L.

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ABSTRACT

Probiotics are viable, non-pathogenic microorganisms which when administered in adequate amounts, confer a health benefit on the host. Mulberry leaves supplemented with *Saccharomyces cerevisiaen* (yeast) and *Bifidobacterium bifidum* (bacteria) probiotics were used to feed two silkworm hybrids. The impact of micro-organisms administration was studied on larval, pupal and cocoon and shell weights. As well as, ERR, cocooning, pupation and cocoon shell percentages. Silk filament length, breaks and silk % were recorded. Digestive enzymes (Protease, Invertase and Amylase) were estimated colorimetricaly. The results revealed that, *B. bifidum* and *S. cerevisiae* improved most tested parameters comparing with control. The effect of probiotics may be dependent on the tested *Bombyx mori* hybrid. Renditta that stands for the quantity of cocoons required for producing a kilogram of raw silk was significantly improved in all supplemented groups either for *B. bifidum* or *S. cerevisiae*. The lowest cocoon kilograms required to produced one kilo of raw silk was (5.97±1.85) recorded for hybrid 2 treated with *B. bifidum*.There is a pronounced increase in the activity of protease, amylase and invertase in probiotic treated worms than control.

INTRODUCTION

Mulberry leaf (*Morus* species) is the only food source for silkworm, *Bombyx mori* L. nutrition. The growth, development of larva and subsequent cocoon production are greatly influenced by nutritional quality of mulberry leaves. Supplement mulberry leaves by using different nutrients and feeding to the silkworms are a useful modern technique to increase economic value of cocoons (Masthan *et al*., 2011). Nutritional study on silkworms is an essential requisite for its proper commercial exploitation and is sole factor which augment quality and quantity of silk (Laskar and Datta, 2000).

Probiotics have been defined as "live microbial feed supplements which beneficially affect the host animal by improving its intestinal microbial balance" (Fuller, 1989). According to Charles (2004) lower animals do not have well developed humoral immunity and immune-stimulation could be achieved easily through probiotics. As well as, Amala *et al*. (2011) insist on up-gradation of the immunity of silkworms by probiotics rather than giving control measures for a disease.

Probiotic bacteria are gram- bacteria that produced lactic acid and resistant to

the enzymes in the intestine (e.g. lysozyme) and have the ability to resist the digestion in the intestinal tract, moreover, these bacteria may have an antimicrobial activity (Kimoto *et al.,* 2000). Usage of probiotics is more common in human, and veterinary medical sciences, while there are little reports on the availability of probiotics formulations specifically designed for silkworms (Brigidi *et al.,* 2001).

Saccharomyces cerevisiae has a great commercial value in baking, brewing, distillery industries and as a source of enzymes. Different types of *Saccharomyces* sp. showed potential benefits to the host and used as bio-therapeutic agents (Vargao and Maraz, 2002). It produces several peptides and other compounds able to exert immune and non-immune effects of potential clinical importance (Canani *et al*., 2011).

Bifidobacteria have been studied for a long time, and were first identified in 1899 (by Henry Tissier at the Pasteur Institute, Paris). *Bifidobacteria* are found naturally in the intestines of breast-fed infants at very high levels. A recent review concluded that the "*Bifidobacterium* genus" is certainly the safest among all genera (Meile *et al*., 2008). They are rod-shaped, slim, and with slightly bulbous or clubbed ends. When nutrients are short, *bifidobacteria* tend to fork at one or both ends. These split ends give the bacterium its name, from the Latin word bifidus, meaning 'split in two' (Hoover, 2000).

Enzymes are specific proteins that catalyst biochemical reactions inside any living organism. Enzymes play a significant role in food digestion which in turn influences the growth, development and resistance to disease in silkworm and subsequently enable the silkworm to have a better survival. The digestibility of silkworm larva depends upon the activity of amylase enzyme. It is the key enzyme involved in digestion and carbohydrate metabolism in insect (Esaivani *et al*., 2014).

Therefore, the aim of the study is to investigate the impact of addition of yeast (*Saccharomyces cerevisiae*) and bacteria (*Bifidobacterium bifidum*) probiotics on mulberry leaf for silkworm rearing. As well as, an approach for strengthen *B. mori* immunity to resist the microbial pathogenic attack and to promote cocoon yield.

MATERIALS AND METHODS

Insect:

Disease-free eggs of univoltine silkworms of two different hybrids were imported from Thailand. Rearing was carried out under hygienic conditions according to Krishnaswami (1978). The young larvae (1st~3rd instars) were reared at 27~28°C with 85%~90% relative humidity and the late age larvae (4^{th} and 5^{th} instars) were maintained at 24~26°C with a relative humidity of 70%~80%. At the beginning of the fifth larval instar, each hybrid was divided into three groups in four replicates with 100 larvae/ replicate. Known weights of fresh mulberry leaves were dipped in 50 ml of *Saccharomyces cerevisiae* soln. 10^9 cfu /ml (group 1). The second group was dipped in *Bifidobacterium bifidum* soln. 10^9 cfu /ml. The third group was dipped in distilled water as control. Mulberry leaves (*Morus alba* var. *Kanava* 2) treated with 'Probiotics' were given to grown silkworm larvae every 48 hours then larvae were fed on untreated leaves for the rest of the feeding period.

Spinning larvae were collected manually and mounted in plastic collapsible mountages. Observations on 5^{th} larval weights, effective rate of rearing, pupal weight, cocooning percentage, single cocoon and shell weights, single cocoon shell ratio and Renditta were recorded. Renditta (which recently, Central Silk Technological Research Institute ,CSTRI, in India have given certain constants that can be used for estimating the renditta from the shell ratio. It stands for the quantity of cocoons

required for producing a kilogram of raw silk. The constants suggested by them are, 165 for cocoon with shell ratio of 14-16%, 150 for cocoon with shell ratio of 17-20%, 133 for cocoon with shell ratio of 21-23%) were recorded ;

$$\text{Renditta} = \frac{\text{Constant}}{\text{Shell Ratio}}$$

Technological parameters such as; filament length, number of breaks and silk percent were estimated.

Microbial isolates and growth media:

Saccharomyces cerevisiae was provided by the Culture Collection of the Regional Center for Mycology and Biotechnology (RCMB), Al-Azhar Uni., Cairo, Egypt. It was cultured at 25 °C for 24 h in yeast-peptone-dextrose (YPD) broth containing 0.5% (w/v) yeast extract, 1% peptone and 2% glucose was used to maintain the cultures. Prior to the experiment, the cultures were activated for 72 h at 25°C and sub cultured at least three times. The total viable cells were adjusted to about 10^9 cfu /ml.

Bifidobacterium bifidum ATCC 15696 was secured from Cairo Microbiological Resources Center (Cairo MIRCEN), Faculty of Agriculture, Ain Shams University and cultured at 37°C for 48 h in 54 g modified MRS (m-MRS) broth which containing of : Peptone 10 g; meat extract 10 g; yeast extract 5 g; potassium mono-hydrogen phosphate 2 g; ammonium citrate 2 g; glucose 20 g; tween (80) 1 ml; sodium acetate ($3H_2O$) 5h; salt solution 5 ml; distilled water 1000 ml; pH 6.2-6.6. Salt solution: $MgSO_4.7H_2O$, 11.5 g; $MnSO_4$. $2H_2O$, 2.4 g; distilled water, 100 mol. supplemented with 0.05% L–Cysteine HCL and 0.3% lithium chloride. Ingredients were dissolved in distilled water; pH was adjusted to 6.5 before sterilization at 121°C for 20 minutes.

Biochemical analysis:

Fifth instar larvae (7^{th} day old) were homogeniezed in distilled water (50 mg/ 1ml). Homogenates were centrifuged at 8000 rpm for 15 min at 5 ^oC in a refrigerated centrifuge. The deposits were discarded and the supernatant were kept in a deep freezer till use.

Protease enzyme:

Proteolytic activity was measured as described by Tatchell *et al.* (1972), with some modifications, by measuring the increase in free amino acids split from substrate protein (albumin), during one hour incubation at 30◦C. The reaction mixture consisted of 100 ul homogenate and 1 ml of 0.1 M phosphate buffer (pH 8) and 100 ul of 0.5 % bovine serum albumin. The reaction was stopped by adding 1.2 ml 20% TCA (trichloro-acetic acid). After standing for 15 min, the mixtures were centrifuged at 3000 rpm for 20 min, and the supernatant was used for measuring the produced amino acids. Amino acids were colorimetricaly assayed by ninhydrin reagent according to the method described by Lee and Takabashi (1966). The reaction mixture consisted of 100 ul supernatant; 1.9 ml of ninhydrin-citrate (pH 5.5); 0.2 ml of 0.5 M citrate buffer (pH 5.5) and 1.2 ml glycerol. The mixture was heated in boiling water bath for 12 min then cooled by tap water. The developed color was read at 570 nm. Zero adjustment was against reagent blank containing the same reaction mixture and 100 ul distilled water instead of the supernatant, L alanine was used as the standard and the amino acids were expressed as ug alanine /min/g.b.wt.

Amylase and Invertase enzymes:

Amylase and Invertase enzymes were determined according to the modifications of Amin (1998) to the method described by Ishaaya and Swirski

(1976).

20 µl of diluted Amylase solution was incubated for 10 min at 30˚c with 250 µl 1% starch (soluble potato starch, Lintner grade, Sigma Chemical Co.) in 50 mM acetate buffer pH 5.0 containing 20 mM NaCl and 0.1mM $CaCl_2$.

20 µl of diluted Invertase solution was incubated for 10 min at 30 ˚c with 250 µl 4% sucrose solution and 230 ul phosphate buffer (pH, 5.4, 0.1 M).

The reaction was stopped by adding 250 µl DNS reagent to each tube in boiling water for 5 min. Samples were cooled, diluted with 2.5 ml H_2O, and read at 550 nm on Spectronic 1201 (Beckman , USA). Glucose was used as a standard. Enzyme activities were expressed as µg glucose released /min/gm fresh weight.

Statistical analysis

Collected data were recorded and analyzed using statistical analyzing system version 9.1 program proc. GLM (SAS Institute, 2003).

RESULTS AND DISCUSSION

Mulberry leaves are complete diet for silkworm but some time it is possible that some deficiencies occur due to different reasons, so supplementation with useful and cheap extra nutrients and feeding to the silkworms is a applicable technique to increase silkworm rearing outputs.

As represented in Table (1) control hybrid 2 showed higher ERR than control hybrid 1(81±10.18 and 71±4.23 %, respectively). Administration of *Bifidobacterium bifidum* increased significantly ERR and recorded 89±6.67 for hybrid 1 and 90±8.7 for hybrid 2. As well as, *Saccharomyces cerevisiae* increased significantly ERR to 92±3.65 for hybrid 2 and 91±5.78 for hybrid 1.

Table 1: Effect of *Bifidobacterium bifidum* and *Saccharomyces cerevisiae* food supplementation on *B. mori* hybrids performance.

	Hybrid 1			**Hybrid 2**			
	Cont	***Bifidobacterium bifidum***	***Saccharomyces cerevisiae***	Cont	***Bifidobacterium bifidum***	***Saccharomyces cerevisiae***	LSD 1%
ERR	71±4.23 b	89±6.67 a	91±5.78 a	81±10.18 ab	90±8.7 a	92±3.65 a	13.7
Larval wt	2.66±0.0 c	3.02±0.02 b	3.05±0.1 ab	2.69±0.05 c	3.07±0.01 a	3.05±0.05 ab	0.04
Cocooning	72±15 b	88±20 a	90±11 a	74±7.5 b	92±4.65 a	96±2.52 a	11.13
Pupation %	60.00±8 c	72.86±6.66 bc	96.29±5.04 a	68.76±8.6 c	78.79±9 b	97.34±6.51 a	9.2
Pupal wt	1.02±0.1 a	1.16±0.04 a	1.32±0.02 a	1.03±0.06 a	1.25±0.02 a	1.19±0.08 a	0.39

Letters in same raw represent the significancy at $P < 0.01$

Larval weight was significantly increased for all treated groups, the best results were recorded for hybrid 2 treated with *B. bifidum* (3.07±0.01 gm) followed by S. *cerevisiae* for both hybrids 1 and 2 (3.05 gm).

Administration of *B. bifidum* and S. *cerevisiae* significantly improved cocooning percentages comparing with control. The highest percentage was recorded for S. *cerevisiae* hybrid 2 (96±2.52 %). The same trend was obvious in pupation percentage, S. *cerevisiae* recorded highest percentages for both hybrids 1 and 2 (96.29±5.04 and 97.34±6.51%, respectively).

Pupal weight was not significantly increased in all administrated groups; the highest value was recorded for S. *cerevisiae* hybrid 1 (1.32±0.02 gm).

The economics of silk production is the crucial factor in evolving sericulture programme for farmers; it is based on the following main factors:- (a) Conversion of mulberry leaves to cocoons; the weight parameters. (b) Quality of filament and its length in the cocoon. (c) Renditta-the proportion by weight of reeled raw silk in a cocoon.

Generally hybrid 2 showed highest values in all tested parameters. Single cocoon characters were statistically analyzed and represented in Table (2). The results revealed that, there were not significant changes in the single cocoon weight among tested groups. Single cocoon shell weight, S. *cerevisiae* administration recorded the highest value significantly (0.34±0.01 gm) for hybrids 2.

Table 2: Effect of *Bifidobacterium bifidum* and *Saccharomyces cerevisiae* food supplementation on *B. mori* hybrids technological parameters.

	Hybrid 1			**Hybrid 2**			
	Cont	***Bifidobacterium bifidum***	***Saccharomyces cerevisiae***	Cont	***Bifidobacterium bifidum***	***Saccharomyces cerevisiae***	LSD
Single Cocoon weight (gm)	1.18±0.05 a	1.43±0.04 a	1.60±0.03 a	1.30±0.07 a	1.50±0.09 a	1.55±0.08 a	0.47
Single cocoon shell weight (gm)	0.21±0.02 b	0.31±0.01 ab	0.32±0.01 ab	0.24±0.01 b	0.33±0.03 ab	0.34±0.01 a	0.09
Single cocoon shell %	17.80±2.02 d	21.68±1.44 b	20.03±1.07 c	18.36±1.77 d	22.11±0.71 a	21.92±1.38 ab	0.64
Renditta	8.42±2.2 a	6.22±1.35 b	7.48±1.4 ab	8.25±2.4 a	5.97±1.85 b	6.15±1.55 b	2.68
Silk filament length	710±28.47 c	884±62.07 ab	759±33.15 bc	781±26.76 bc	923±70.77 a	866±41.2 ab	128
Silk filament breaks	3±1 b	0 a	1 ab	2±1.53 b	0 a	0 a	2.04
Silk %	12±1.53 b	15.68±1.47 a	13.24±1.03 ab	12.03±0.79 b	15.87±0.75 a	14.29±0.62 ab	2.68

Letters in same raw represent the significancy at $P < 0.01$

Single cocoon shell ratio of *B. bifidum* resulted in significantly highest percentages for hybrid 2 (22.11±0.71 gm).

Renditta that stands for the quantity of cocoons required for producing a kilogram of raw silk, was significantly improved in all supplemented groups either for *B. bifidum* or *S. cerevisiae* for both hybrids 1 and 2. The lowest cocoon kilograms required to produced one kilo of raw silk was (5.97±1.85) recorded for *B. bifidum* of hybrid 2. Silk filament and raw silk percentage were 923±70.77 m and 15.87±0.75 % for *B. bifidum* hybrid 2.

Masthan, *et al*. (2011) found that, the treatment with 300 ppm concentration yeast is significant in increasing the cocoon characters; single cocoon weight, single shell weight, pupal weight and silk filament length when compared with control. Secondly, the differences between 300 ppm concentration and other two concentrations namely 100 and 200 ppm are also significant. The differences between 100 and 200 ppm concentrations are not found to be significant except for filament length. Other attempt was performed with *Lactobacillus plantarum* probiotic which improves the cocoon production of mulberry silkworm, *Bombyx mori* (Singh *et al.*, 2005). Certain probiotic bacteria inhibit the growth of microbes; *Streptomyces noursei* are probiotic microbes which prove the antibacterial activity and good eco-friendly management of silkworm diseases (Subramanian *et al*., 2009).

It may be concluded that the cocoon characters and silk filament were improved upon the effect of *B. bifidum* and *S. cerevisiae* probiotics significantly and the effect is more obvious with *B. bifidum* on hybrid 2. The effect of probiotics is dependent on hybrid as clarified for *S. cerevisiae* effect on hybrid 2 was nearly showed the same results as *B. bifidum* on hybrid 1.

Table (3) represent the digestive enzymes (Protease, Invertase and Amylase)

activities of larvae feed on mulberry leaves supplemented with *B. bifidum* and *S. cerevisiae*. Protease recorded highest activity upon the effect of *S. cerevisiae* on hybrid 1 (70.77±3.65 ug D,L-alanine/min/g.b.wt) followed by *S. cerevisiae* on hybrid 2 (62.1±2.95 *ug D,L-alanine/min/g.b.wt)*. Highest invertase activity was recorded for hybrid 2 administrated with *S. cerevisiae* (494.67±6.11ug glucose/min/g.b.wt). And the highest amylase activity was recorded for hybrid 1 supplemented with *S. cerevisiae* (27.77±1.59 ug glucose/min/g.b.wt).

There was a profound increase in the activity of the amylase and invertase in the digestive juice of the probiotic treated with *Saccharomyces cerevisiae* worms than the control as reported by Esaivani *et al*. (2014) which support the present findings.

Table 3: Digestive enzymes analysis of *B. mori* hybrids upon the effect of *Bifidobacterium bifidum* and *Saccharomyces cerevisiae* food supplementation.

	Hybrid 1			**Hybrid 2**			
	Cont	***Bifidobacterium bifidum***	***Saccharomyces cerevisiae***	Cont	***Bifidobacterium bifidum***	***Saccharomyces cerevisiae***	LSD
Protease *(ug D,L-alanine/min/g.b.wt)*	28.7±2.60 e	52.77±2.25 cd	70.77±3.65 a	44.9±3.54 d	56.87±2.72 cb	62.1±2.95 b	8.21
Invertase *(ug glucose/min/g.b.wt)*	346.33±7.57 c	361±6.81 c	428±15.62 b	352±12.17 c	436±10.44 B	494.67±6.11 a	28.35
Amylase*(ug glucose/min/g.b.wt)*	13.87±0.7 c	23.97±1.54 b	27.77±1.59 a	16.57±1.6 bc	25.7±1.3 Ab	26.5±1.14 ab	3.7

Letters in same raw represent the significancy at $P < 0.01$

The main by-products of *bifidobacteria* metabolism are acetic acid and lactic acid, in about equal proportion. These two acids lower the pH (increase acidity) within the intestine. It is likely that the ability of *bifidobacteria* to increase the acidity of the intestine is a factor in their probiotic effects, as many harmful microbes are inhibited in a low pH environment (Rasic and Kurmann, 1983). There is also some evidence that *bifidobacteria* produce anti-bacterial substances that inhibit harmful bacteria to adhere to intestinal wall (Biavati *et al*., 2000). Other useful characteristics of *bifidobacteria* are the production of various B vitamins, and a tendency to adhere well to the intestinal wall, thus excluding pathogenic bacteria (Bernet *et al*.,1993). Riboflavin, vitamin B2, enhances silk production when sprayed on mulberry leaf and feeding to silkworm (Ito, 1978).

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ARABIC SUMMERY

الكائنات الحية الدقيقة كمكملات غذائية ليرقات الحرير التوتيه

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الكائنات الحية الدقيقة (Probiotic) هي كائنات مجهريه غير مسببة للأمراض عندما تضاف بكميات كافية إلى الطعام ، تساعد في رفع كفاءة الإستفاده من الطعام و بالتالي تحسين الحالة الصحية للجسم.
أستخدمت في هذه الدراسه الخميرة *Saccharomyces cerevisiaen* و بكتيريا الألبان *Bifidobacterium bifidum* لتغذية هجينين مختلفين من يرقات الحرير التوتيه بإضافتها على ورق التوت.
تم دراسة تأثير هذه الكائنات الحية الدقيقة على وزن اليرقات ، العذراء ، الشرنقة وغلاف القشرة . وكذلك ، كفاءة معدل التربية , نسبة التعشيش ، نسبة التعذير ، نسبة وزن غلاف الشرنقة إلى وزن الشرنقة . أيضا دراسة خواص خيط الحرير ؛ طوله و عدد مرات قطع الخيط أثناء عملية الحل و نسبة الحرير الخام الناتج من كل شرنقة . تم تقدير إنزيمات الهضم (Protease, Invertase and Amylase) كميا.
أسفرت النتائج أن كلا من البكتيريا و الخميرة حسنت معظم القياسات التي تم إختبارها مقارنة مع الكنترول . ومن المرجح أن تأثير هذه الكائنات يختلف بإختلاف الهجين المستخدم . Renditta و التي تشير إلي كمية الشرانق المطلوبة لإنتاج كيلوجرام واحد من الحرير الخام تحسنت بشكل ملحوظ في جميع المجموعات المعاملة . الهجين ٢ المعامل بالبكتيريا نتج عنه أقل كيلوجرامات من الشرانق المطلوبة لإنتاج كيلوجرام واحد من الحرير الخام (5.97 كيلو جرام). هناك زيادة واضحة في نشاط إنزيمات الهضم في الديدان المعاملة بهذه الكائنات الحية الدقيقة مقارنة بالكنترول .