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Effect of *Azolla* on total Haemocyte count in two various races of Silkworm, *Bombyx mori* (L.) reared on G4 mulberry foliage

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Abstract
Haemocytes are the free cells in the haemolymph of silkworms. The overall haemocyte amount specifies the immunological stage of an insect. The THC varies during the development and metamorphosis of various races of the silkworm. The THC of a double hybrid bivoltine (FC1×FC2) race changed into in comparison with that of single hybrid bivoltine (CSR2×CSR4) in this work. In the present study, the highest THC value was found at 3rd day of 20% *Azolla* treated IV instar larvae in two races under study, but a higher count was found in FC1×FC2 (8533±611 cells/mm^3) than CSR2×CSR4 (5493±646 cells/mm^3). Further, an increased value of THC was found in 5th day of 20% *Azolla* treated V instar larvae of FC1×FC2 (19626±402 cells/mm^3) than CSR2×CSR4 (18773±360 cells/mm^3). Two-way ANOVA observed by means of Dunnett's multiple comparisons check results indicate the significant differences between the two races in terms of THC.

Keywords: Azolla, bivoltine, Haemocytes, Silkworm, Total haemocyte count

Introduction
Bombyx mori L, commonly called to as the domestic silkworm (Mita., 2004) [15] or mulberry silk worm (Mohanta., 2010) [16]. The silkworm is a monophagous insect that depends on the mulberry for its whole growth and development (Manu., 2020) [13]. *Bombyx mori* is an important commercial insect, is being used as a tool to transform mulberry leave protein into silk (Simi Simon *et al*., 2024) [5]. The silk product is dependent on the larval nutrition and nutritional value of mulberry leaves, which performs a veritably vital function in producing good-quality cocoons (Legay., 1958) [12]. The different types of silkworm change in their nutritive value, metabolic and physiological process (Ravikiran *et al*., 2024) [25]. The FC1×FC2 double hybrid bivoltine can repel adverse climatic conditions and performing in crop balance than single hybrid bivoltine. CSR2×CSR4 productive bivoltine sorts with high survival and cocoon parameters. Mulberry leaves choice is an crucial factor for successful silkworm parenting (Md Habibullah Bahar *et al*., 2011) [14]. To increase the yield and to suit different regions of the country, numerous bettered kinds are being evolved, among them G4 variety shows high resistance to pest, survival, sprouting and establishment characteristics than V1 variety (Sudhakar *et al*., 2019) [27]. In addition to mulberry leaves, feed supplements also are given to silkworm to enhance profitable characteristics (Jeyapaul., 2003 [6]; Sheeba., 2006) [24]. The free floating fern *Azolla*, which belongs to the family Azollaceae which is a good source and protein contains nearly all essential amino acids, minerals and reasonable quantities of vitamin precursors beta- carotene and vitamin B12 (Abdalbakee *et al*., 2019) [1]. Silkworms have an open circulatory system containing haemolymph. It comprises of fluid which has blood cells i.e., haemocytes, water, fat, carbohydrates, proteins etc. Haemolymph is the only extracellular fluid containing the products needed for every physiological exertion of the insect body. Therefore, changes in the composition of haemolymph reflect the physiological and biochemical metamorphosis taking place in the insect tissues (Ponmurugan *et al*., 2017) [21]. The haemocyte present in haemolymph performs physiological functions like phagocytosis, encapsulation, detoxification, storage and distribution of nutritional supplements. Haemocytes play a pivotal a part of scavenging, and their viscosity alters during transformation (Wigglesworth.,1973; Yamashita *et al*., 2001) [30]. As alteration in structure, types and number of cells reflect changes in physiological and natural processes (Navanita Bhagawati *et al*., 2012) [17]. The present study is concerned with the total haemocyte count (THC) of two different silkworm races fed with G4 mulberry leaves treated with *Azolla*.

Materials and Methods
Silkworm rearing
The present study was performed at the Research Laboratory of Zoology, Tumkur University, Tumakuru. FC1×FC2 and CSR2×CSR4 races of silkworms have been raised under laboratory conditions of 25±3°C, 72±2% relative humidity. The larvae were divided into 3 sets; each set has 20 larvae, which were supplied with an adequate amount of G4 mulberry leaves. The leaves were always cleaned and washed from dust and given to the IV and V instar larvae, at two intervals. The larvae were reared under preferred rearing conditions (Krishnaswami, 1973) [9].

Mulberry leaves treated with *Azolla* supplement
Dried *Azolla* was bought from Biogreen Agri Links, Bengaluru. *Azolla* was powdered using a mixer and made into different concentrations (5%, 10%, 15%, and 20%) and treated with mulberry leaves, and fed to silkworms twice a day from the fourth instar first day until they reached the spinning stage.

Determination of THC
Total haemocytes count was done using the method described by the (Praful., 1994) [20]. For the haemocytes

study, the haemolymph was obtained from the IV and Vinstar larvae of FC1×FC2 and CSR2×CSR4 races of silkworm *Bombyx mori* (L.). THC was estimated by Neubauer haemocytometer. The healthy, well-fed larvae of IV and V instars were taken. The larvae (2 larvae from each set) were kept in hot water at 50℃ for 2 minutes to fix the haemolymph. After fixation, the larvae are removed and dried using filter paper. Then the haemolymph was collected by cutting the metathoracic proleg by scissor, and the blood was drawn by an insulin syringe of 2 ml. The haemolymph was collected in sterilized, clean, and precooled Eppendorf tubes containing 0.0002g of phenylthiourea (to prevent the oxidation of haemolymph). The haemolymph is drawn up to mark 0.5 of a WBC diluting pipette, then the tip is cautiously wiped clean and immediately diluted in WBC diluting fluid up to mark 11 and shaken vigorously for several minutes. The first 3 drops are discarded. Then the samples was transferred to a clear counting slide, and covered with a cover slip, and observed below microscope. While making the observation of the THC, five squares of 1 mm size (4 big corners and central big squares) were counted for the calculation of total haemocytes (THC) as suggested by (Jones., 1962 [7]; Jalali *et al*., 2008) [5]

$$THC = \frac{\text{Haemocytes in five } 1mm^2 \times \text{Dilution} \times \text{Depth factor of the chamber}}{\text{Number of squares counted}}$$

Dilution – 20 times
Depth – 0.1
No. of squares counted – 05

Statistical Analysis
The given data were analyzed by a Two-way ANOVA observed by means of Dunnett's multiple comparisons check, using a commercially available statistics software package (Graphpad Prism 8 for Windows).

Results
The haemolymph of the silkworms FC1×FC2 and CSR2×CSR4 races is yellow coloured. The prevailing observe found out that the supplementation of *Azolla* with G4 mulberry leaves for silkworm *Bombyx mori* FC1×FC2 and CSR2×CSR4 exhibited significant results on Total Haemocyte Count (THC) in IV and V instars. This result indicates that a G4 mulberry leaves with *Azolla* show a better outcome in all stages of silkworm.

Total Haemocytes Count (THC)
IV Instar: The THC was carried out in haemocytometer every day of IV and V instars of FC1×FC2 and CSR2×CSR4 silkworm races. Compared to IV instar larvae V instar larvae have the highest haemocyte count. In Table 1(a) and Fig 1(a), the studies showed that the THC of FC1×FC2 silkworm was IV instar. The THC value gradually increased each day until it reached its maximum on 3rd day and declined on the molting day. 20%, 15%, 10%, and 5% *Azolla* treatments of FC1×FC2 showed a statistically high significant P value<0.0001 compared to control. Data in Table 1(b) and Fig 1(b) shows that the THC value of 20% *Azolla* treatments of CSR2×CSR4 indicates a statistically good significant P value<0.001, 15% *Azolla* treatments showed statistically good significant P value<0.001, 10% *Azolla* treatments showed a statistically significant P value<0.01, 5% *Azolla* treatments were not significant compared to the control. The data of the investigation shows the total haemocyte count is highest on the 3rd day of IV instar silkworms treated with 20% *Azolla* and least in control. The THC tends to increase former to each ecdysis, then decreases suddenly at ecdysis and increases again shortly afterwards.

V Instar: A similar trend of THC was found in V instar FC1×FC2 and CSR2×CSR4 silkworms. Data in Table 2(a) and Fig 2(a) show there is a gradual increase in THC daily until the 5th day of the V instar FC1×FC2 silkworm race and progressively decreases prior to spinning as the larva lowered the proper feeding. 20% *Azolla* treatments and 15% *Azolla* treatments showed a statistically high significant P value<0.0001 with that of control and 10% *Azolla* treatments showed statistically good significant P value<0.001 with that of control, and 5% *Azolla* treatment showed statistically significant P value<0.05 with that of control. In Table 2(b) and Fig 2(b), shows there is a gradual increase in THC daily until the 5th day of the V instar CSR2×CSR4 silkworm race and progressively decreases prior to spinning, as the larvae lowered the proper feeding. 20% *Azolla* treatments and 15% *Azolla* treatments showed a statistically high significant P value<0.0001 with that of control and 10% *Azolla* treatments showed a statistically good significant P value<0.001 with that of control and 5% *Azolla* treatments were not significant with that of the control. THC reaches a maximum in the pre-pupae. The THC declines very hastily at pupation and ultimately falls to a maximum level during the pupal stage, which is the non-feeding duration.

Table 1a: Effect of different concentrations of *Azolla* on THC in FC1×FC2 race of *Bombyx mori*. L. The values are expressed in Cells/mm3± sd during IV instar.

No. of Days	Total Haemocytes count (cells/mm^3± sd) in FC1×FC2 IV Instar				
	Control	5% *Azolla*	10% *Azolla*	15% *Azolla*	20% *Azolla*
1	3733±611	4533±611 ****	5200±800 ****	5733±1006 ****	6400±1058 ****
2	5066±611	6000±400 ****	6933±611 ****	7066±230 ****	7866±230 ****
3	5466±611	6666±230 ****	7200±400 ****	7733±611 ****	8533±611 ****
4 (Molting)	4933±1006	6000±800 ****	7066±611 ****	7333±230 ****	8000±400 ****

**** P Value < 0.0001 (highly significant)

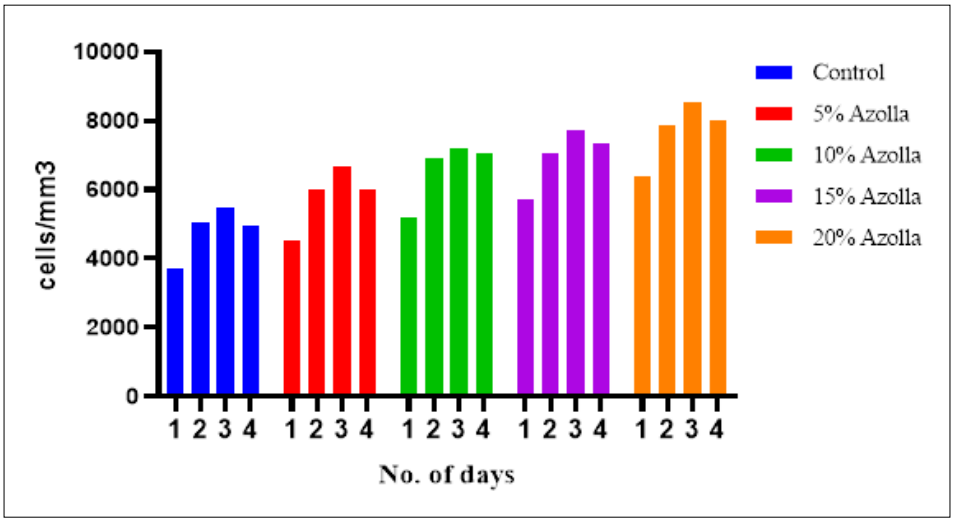

Fig 1a: THC in FC1×FC2 race of *Bombyx mori*. L treated with *Azolla* during IV instar

Table 1b: Effect of different concentrations of *Azolla* on THC in CSR2 × CSR4 race of *Bombyx mori*. L. The values are expressed in cells/mm^3 for THC during IV instar.

No. of Days	Total Haemocytes count (cells/mm^3± sd) in CSR2 × CSR4 IV Instar				
	Control	**5% *Azolla***	**10% *Azolla***	**15% *Azolla***	**20% *Azolla***
1	2693±257	2973±234 ns	3120±174 *	3240±405 **	3533±305 ***
2	2826±83	3066±241 ns	3400±200 *	3613±140 **	3840±211 ***
3	3066±83	3506±272 ns	4413±420 *	4866±503 **	5493±646 ***
4 (Molting)	2946±220	3200±249 ns	3613±371 *	3840±144 **	4000±120 ***

ns- No significant, *P Value < 0.01(significant), ** P Value < 0.005 (significant), *** P Value < 0.001 (good significant)

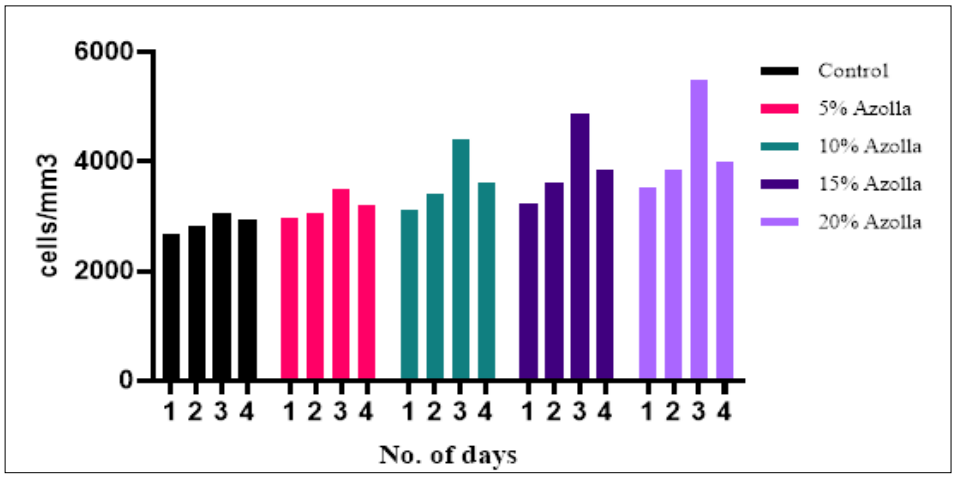

Fig 1(b): THC in CSR2×CSR4 race of *Bombyx mori*. L treated with *Azolla* during IV instar

Table 2a: Effect of different concentrations of *Azolla* on THC in FC1×FC2 race of *Bombyx mori*. L. The values are expressed in Cells/mm3± sd during V instar.

No. of Days	Total Haemocytes count (cells/mm^3± sd) in FC1×FC2 V Instar				
	Control	**5% *Azolla***	**10% *Azolla***	**15% *Azolla***	**20% *Azolla***
1	10266±832	12207±1765 *	14506±725 ***	15280±288 ****	16040±802 ****
2	13746±711	14949±286 *	15666±702 ***	16756±544 ****	18134±546 ****
3	14850±461	16033±404 *	16842±280 ***	17800±480 ****	18493±400 ****
4	15246±430	16520±484 *	17080±338 ***	18000±605 ****	19616±495 ****
5	16277±670	17344±482 *	18074±810 ***	18866±460 ****	19626±402 ****

6	14149±218	14376±345 *	14600±200 ***	14800±320 ****	15084±247 ****
7	14040±990	14313±652 *	14521±456 ***	14601±394 ****	15012±222 ****

*P Value < 0.05(significant), *** P Value < 0.001 (good significant), **** P Value < 0.0001 (High significant)

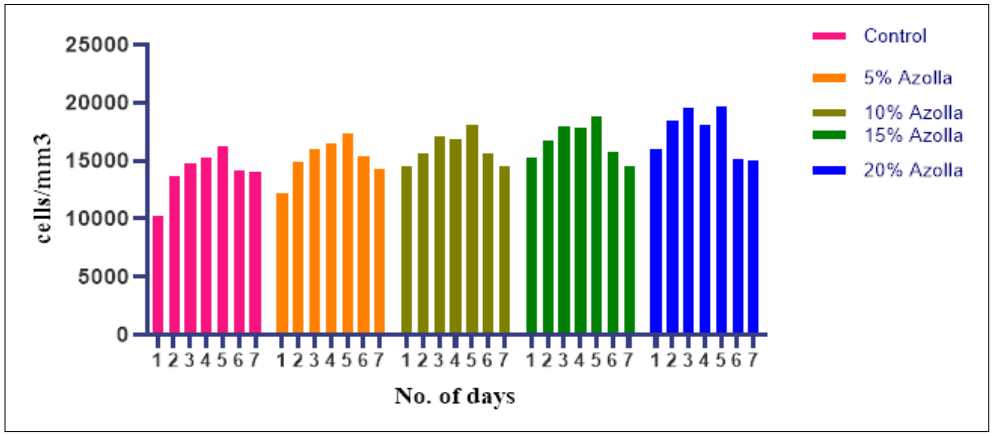

Fig 2(a): THC in FC1×FC2 race of *Bombyx mori*. L treated with *Azolla* during V instar

Table 2b: Effect of different concentrations of *Azolla* on THC in CSR2×CSR4 race of *Bombyx mori*. L. The values are expressed in Cells/mm3± sd during V instar.

No. of Days	Total Haemocytes count (cells/mm^3± sd) in CSR2 × CSR4 V Instar				
	Control	**5% *Azolla***	**10% *Azolla***	**15% *Azolla***	**20% *Azolla***
1	10000±0.57	10160±211 ns	10840±461 ***	12053±323 ****	13533±503 ****
2	10333±416	11653±410 ns	12840±461 ***	14560±654 ****	15880±243 ****
3	11880±589	13480±628 ns	15626±408 ***	17266±440 ****	18413±420 ****
4	13640±802	15680±423 ns	16560±277 ***	17333±384 ****	18453±323 ****
5	15493±257	16386±400 ns	16720±280 ***	17693±546 ****	18773±360 ****
6	13653±967	13760±211 ns	14253±220 ***	14546±220 ****	14960±211 ****
7	12240±654	12813±380 ns	13373±500 ***	13720±589 ****	14149±258 ****

ns- No significant, *** P Value < 0.001 (good significant), **** P Value < 0.0001 (High significant)

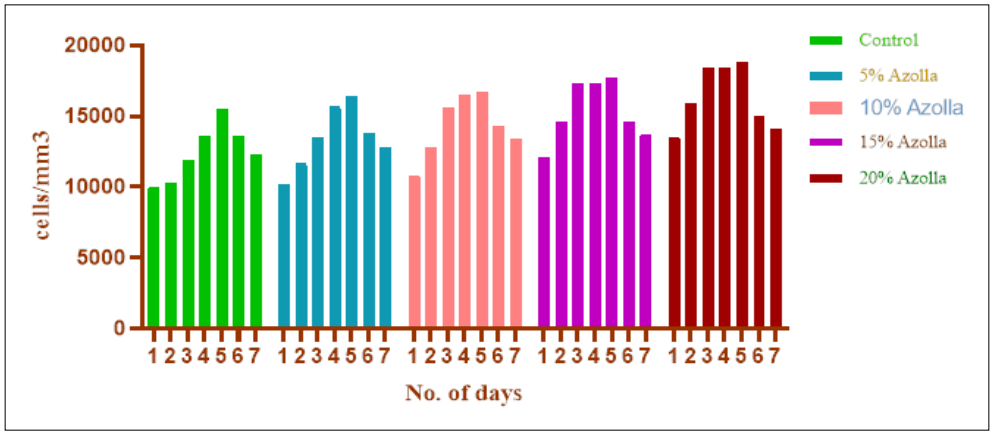

Fig 2b: THC in CSR2×CSR4 race of *Bombyx mori*. L treated with *Azolla* during V instar

Discussion

FC1×FC2 and CSR2×CSR4 races of silkworm, *Bombyx mori* (L.), reared on G4 mulberry leaves treated with distinctive concentrations of *Azolla* exhibit a distinct variance in the total haemocyte count of IV and V instar larvae. These may be due to the different nutritional content of host plants that immediately influences the progress and development of the larvae (Renu Gogoi *et al*., 2022) [23]. Insects do not have an acquired immune system but have a well-developed innate immune system (Lavine and Strand, 2002) [10]. The insect haemocyte is the main component of this innate system that immediately clears pathogens from

circulation (League & Hillyer, 2016) [22]. In the present study, it was observed that THC progressively increased from day 1 until it reached its maximum on the 3rd day and declined on the molting day in the IV instar of FC1×FC2 and CSR2×CSR4 races of silkworm. In V instar larvae, it showed a gradual increase in THC daily until the 5th day of the V instar FC1×FC2 and CSR2×CSR4 silkworm races and gradually decreased prior to spinning as the larva lowered the proper feeding. Silkworm is a holometabolous insect; the rate of THC ranges its peak during the mature stage. The increasing drift ensured better immune resistance to any sort of infection. This could be confirmed by the zero death observed in both races. Greater THC might be due to higher production of cells that give protection to the future quiescent stage of pupa, antimicrobial proteins and proteins required for silk synthesis. The variations discovered between the two races might be attributed to the racial specificity or ingrained genetic character of the specific race. The greater THC of a double hybrid bivoltine and the lower THC of the single hybrid bivoltine races must be their particular characters. These race-specific characters might have been adaptations to environmental situations or seasonal variations, as the earlier works reported (Vinotha *et al*., 2017) [28]. The advanced rate of mitosis that characterizes all other tissues during this duration of dynamic growth may also increase the quantity of haemocytes (Jalal Jalali *et al*., 2008) [5]. Arumugavel *et al*., 2021 [2] reasoned that in *Periplanata americana*, the decrease in THC was due to an increase in haemolymph volume during ecdysis. An increase in THC in *Oncopelitus fasciatus* after molting was reported by (Fier *et al*., 1969) [4]. In *Anagasta Sp,* also Nittono., 1960 observed that the THC was higher in larvae than in the other developmental stages. The haemocyte count has better in *Azolla*-fed larvae. (Prabina *et al*., 2010) [19] Birds given 10% dried *Azolla* had a higher antibody tire value against Ranikhet virus than birds given 7.5% dried *Azolla*. Hence, larval diet is considered one of the cheif factors that determine the abundance of immune cells, which was practically proven by (Siva-Jothy and Thompson, 2002 [26]; Yang *et al*. 2008) [31]. Feeding with a proper food and appropriate care is vital to the cellular abilityof silkworms, as it supplies a defensive mechanism against diseases (Renu Gogoi *et al*., 2022) [23].

Conclusions

In the present study, Total haemocyte count (THC) obtained was compared with the available literature and discussed. This result indicates that G4 mulberry leaves with *Azolla* show a better outcome in all stages of silkworm. The blood volume not only indicates immunity but also maintains the body size of the silkworms. Compared to IV instar larvae V instar larvae have the highest haemocyte count. The data of the investigation shows the total haemocyte count is highest in silkworms treated with 20% *Azolla* and least in control in both races especially in FC1×FC2. This is because FC1×FC2 is a bivoltine double hybrid race that has better seed crop performance, and higher seed recovery, and has the best crop stability. CSR2×CSR4 is a productive bivoltine race with high survival and cocoon parameters. Feeding silkworms with an appropriate diet and good care is vital to their cellular fitness of silkworms, as it provides a defensive mechanism against infections.

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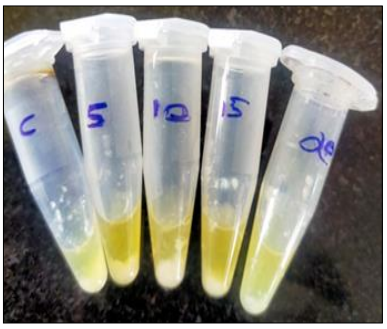
Declaration of competing interest: The author's states that they have no known challenging financial interests that could have seemed to impact the work reported in this paper.

Photos

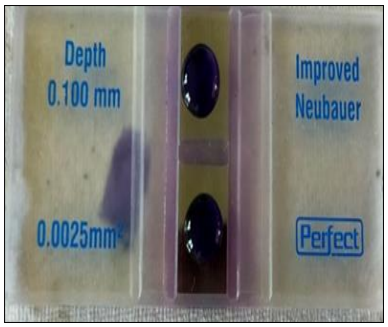
FC1 × FC2 silkworms

CSR2 × CSR4 silkworms

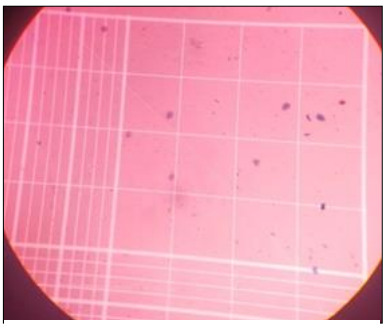


Haemolymph of FC1 × FC2

Haemolymph of CSR2 × CSR4



Neubauer Haemocytometer



Microscopic view of Haemocytes in haemocytometer

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