

# Screening of Environmental Bacterial Strains for Wild *Aedes* Mosquito Larval Biocontrol

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## Abstract

The effectiveness of current control measures against *Aedes* mosquitoes remains low, resulting in persistent epidemics in urban areas. The emergence of resistant mosquito populations to chemical insecticides highlights the need for novel, environmentally friendly, cost-effective control strategies. This study explored the potential of environmental bacterial isolates to biocontrol wild *Aedes* larvae. Initially, we collected bacterial samples from infectious masses of *Aedes fluviatilis* larvae. The isolated bacteria were identified using biochemical, enzymatic, and molecular methods, including 16S rRNA sequencing and MALDI-TOF. Previously, *Aeromonas hydrophila* and *Bacillus thuringiensis* isolated from these infectious masses showed limited *Aedes* larval inhibition. Consequently, we screened additional environmental isolates from the bacteriotheque. Six isolates previously identified were tested: *Chromobacterium violaceum*, *Enterobacter cloacae*, *Bacillus cereus*, *Bacillus sphaericus*, and two strains of *Bacillus thuringiensis israelensis*. Among these strains, *B. thuringiensis* and *C. violaceum* exhibited significant inhibitory activities against wild *Aedes* larvae. *Bacillus thuringiensis* cultures grown under daylight conditions showed a slight ability to inhibit *Aedes* larvae. The potential of *B. thuringiensis* and *C. violaceum* strains studied, along with optimized culture growth conditions, will be further investigated to develop bioinsecticide products to provide safer and more sustainable alternatives for controlling larvae of *Aedes* mosquitoes.

## Keywords

*Bacillus thuringiensis*, *Chromobacterium violaceum*, *Aedes* Biocontrol, Larval Inhibition

## 1. Introduction

Current control measures aim to eliminate this mosquito at various life stages [1]; however, the effectiveness of these interventions has generally been very low, and epidemics continue in urban centers [2]. The systematic use of chemical products to combat mosquitoes may become ineffective due to the emergence of *Aedes aegypti* populations resistant [3]-[5].

New technologies have emerged as important for selective vector control strategies, including using natural products that are less aggressive to the environment, have good results, and are low-cost [6]. Bioinsecticides appear as a response to growing environmental concerns and are efficient in controlling vector insects with minimal environmental damage [4].

Bacteria use in the biological control of mosquito larvae has stood out among the various strategies comprising integrated management programs. The main reasons for this are their advantages over chemical insecticides. These products have numerous formulations and are used against several mosquito species, with a slightly higher price than traditional products. Still, they are competitive when considering the social and environmental costs of using non-selective insecticides in aquatic ecosystems [7].

Many species of bacteria associated with insects are known, but few have desirable characteristics for application in biological pest control. Potentially active bacteria against insects include those that can multiply within the organism, leading to septicemia, or those capable of producing entomotoxins active at any stage of the host's life. According to Falcon's (1971) criteria, entomopathogenic bacteria are grouped into sporulating and non-sporulating. Sporulating bacteria are considered of greater commercial interest in insect biological control because they persist longer in the environment and are easier to produce on an industrial scale. Sporulating bacteria include strict aerobes and most facultative, grouped in the family *Bacillaceae*.

The best-known entomopathogenic bacteria are *Pseudomonas* (*P. aeruginosa*, *P. fluorescens*), *Serratia* (*S. marcescens*, *S. entomophila*), *Clostridium* (*C. bifermentans*), and *Bacillus* (*B. alvei*, *B. larvae*, *B. laterosporus*, *B. lentimorbus*, *B. popilliae*, *B. sphaericus*, *B. thuringiensis*) [8]. Bioinsecticides based on *Bacillus* species have stood out as an alternative for controlling urban and agricultural pests [9], with *Bacillus thuringiensis* responsible for 90% - 95% of the bioinsecticide market [10].

Various laboratories worldwide seek new bacteria isolates that produce different toxins or are better adapted to local conditions to improve field efficacy and potential use in managing insect resistance [11]. The bacterium *Bacillus thuringiensis israelensis* (Bti) produces three different Cry toxins (toxic crystal) and one Cyt toxin (with cytolytic and hemolytic activity). This synergistic action typically occurs when more than one pesticide with different modes of action is used, acting on multiple biochemical and physiological processes and killing the insect in different ways [12]. These large numbers of toxins reduce the likelihood of resistance development [13] [14].

In this study, we investigated the bacterial etiological agents involved in the lethal disease of *Aedes fluviatilis* colonies. Then, we also observed whether these microorganisms and some environmental isolates could inhibit larvae of wild *Aedes* mosquitoes.

## 2. Material and Methods

### 2.1. Sample Collection

The infectious masses containing the bacteria of interest for this study were obtained from 2nd and 3rd instar larvae (**Figure 1**) of an *Aedes fluviatilis* colony maintained in the Entomology Laboratory at the Institute of Biology (IB) of the Federal University of Mato Grosso (UFMT). This colony is well-established and adapted to local conditions but has begun to show contamination issues.



**Figure 1.** Infectious mass at the posterior end of the larval intestine of *Aedes fluviatilis*. This whitish-colored mass hindered the emergence of the larvae to breathe, leading to their death by asphyxiation.

During the experimental period, the colonies were maintained in a climate-controlled chamber with a temperature range of  $28^{\circ}\text{C} \pm 2^{\circ}\text{C}$ , relative humidity above 60%, and a photoperiod of 12 hours. They were kept in treated water (tap water), decanted in a barrel for 48 hours, and fed industrialized fish feed.

The infectious masses were carefully dissected and removed from the larvae in sterile Petri dishes, subjected to 10 washes with sterilized saline solution, and stored in vials with 70% alcohol. They were also frozen in BHI broth with 2% glycerol. Additionally, whole diseased larvae were stored in 70% alcohol.

### 2.2. Bacterial Isolation and Biochemical Characterization

The infectious masses were macerated with sterile saline solution and then inoculated into various culture media, such as tryptic soy broth (TSB), sodium thioglycolate broth, blood agar, nutrient agar, MacConkey agar, eosin methylene blue agar, and selective agars for *Bacillus* spp. The cultures were incubated for 24 to 72 hours at room temperature and  $35^{\circ}\text{C}$ . TSB and thioglycolate tubes showing growth (turbidity) were inoculated in the mentioned agars.

The cultured microorganisms were fixed on slides and stained by the Gram

method. The identification of cocci and bacilli was carried out using biochemical, enzymatic, and molecular techniques. Commercial kits from PROBAC were used for preliminary biochemical characterization. Quality control of the kits for biochemical determinations was performed using the following standard samples: *Escherichia coli* ATCC-25922, *Pseudomonas aeruginosa* ATCC-27853, and *Staphylococcus aureus* ATCC-25923.

Fifty environmental isolates deposited in the bacteriotheque from the Microbiology Laboratory of the Medical Faculty of UFMT were previously tested through bioassays for their ability to kill or inhibit the development of wild mosquito larvae. Through these tests, six strains were selected for classical and molecular identification, as well as for further bioassay studies.

Finally, these microorganisms were identified by 16S rRNA by Sanger sequencing, MALDI-TOF (Biotyper, Bruker), next-generation sequencing, and metagenomic next-generation sequencing (mNGS).

### 2.3. Collection and Development of Wild Mosquito Larvae

The Entomology Laboratory at the Institute of Biology (IB) of the Federal University of Mato Grosso (UFMT) followed a well-established protocol for collecting and maintaining wild mosquitoes. To obtain larvae, oviposition traps, glass beakers (provided by the laboratory), disposable cups, yellow rubber bands, tulle, and rest mineral water were used. The oviposition trap consisted of a black plastic container, an Eucatex palette (**Figure 2**), containing a liquid left to rest for five days in a dark, covered container. This liquid comprised 20.7 grams of hay and 2.5 liters of water, called hay infusion. After five days of resting, the infusion was strained to separate the liquid from the hay. This liquid was poured into the black container, the trap's palette, and 30% mineral water was added, resulting in a mixture of 70% hay infusion and 30% mineral water. The traps were placed in suitable locations, *i.e.*, areas with a higher likelihood of *Aedes aegypti* presence, and left for seven days. They were then collected, with the liquid discarded and the oviposition palettes stored for subsequent hatching in beakers with rested water.



**Figure 2.** Eucatex palette with captured eggs of wild mosquitoes.

### 2.4. Bioassay

Some experiments were adapted to work with wild mosquitoes because the *Aedes fluviatilis* colony maintained in the laboratory was decimated after contamination

with a disease that produced infectious White-masses in the distal portion of the larvae's intestine.

For the bioassays, eggs from wild *Aedes aegypti* colonies were used under previously established conditions adapted to the Entomology Laboratory of the Institute of Biology. During this research, we observed that, interestingly, the eggs captured in oviposition traps, especially in dry periods, had lower vigor, which frequently resulted in a low hatching rate. The bioassays were set up in 400 ml beakers or disposable cups, containing 1 mL of bacterial cultures ( $1 \times 10^8$  cells/mL), 200 mL of rested water, and 20 second-instar larvae after the eggs collected from the traps had hatched (**Figure 3**). Each bioassay was conducted in duplicate to observe the inhibitory efficacy of the bacteria, with a control (no treatment, containing only rested water) and a control with a culture medium used for microbial growth (brain heart infusion broth, BHI). Experiments with bacteria exhibiting potential inhibitory activity were repeated. Another factor analyzed was the cultivation of these microorganisms under both light and dark conditions.

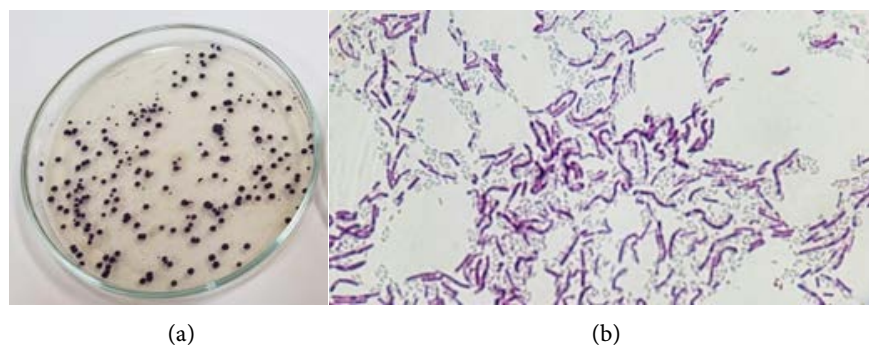


**Figure 3.** Bioassay with wild larvae of *Aedes* mosquitoes

### 3. Results

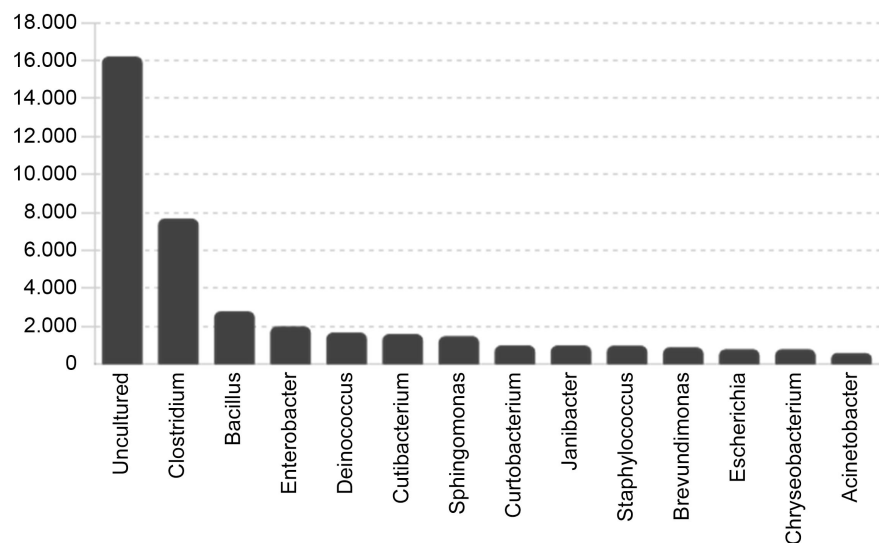
#### 3.1. Identification of Microorganisms Isolated from the Infectious Masses of *Aedes fluviatilis*

Colonies isolated from the infectious masses of *Aedes fluviatilis* from culture plates were replicated onto new culture media to obtain pure cultures. After incubation at 37°C for 24 hours, slides of isolated colonies were prepared and observed fresh and with Gram staining, as shown in **Figure 4**.



**Figure 4.** Prevalence of two bacilli in agar (a) from TSB broth, one Gram-negative and the other Gram-positive growing concomitantly and in an aggregated form (b).

These microorganisms were characterized biochemically, by MALDI-TOF, and through Sanger gene sequencing with universal primers of mRNA 16S and replicases (*rpoB* and *gyrA*). The Gram-negative bacterium was identified as *Aeromonas hydrophila*, and the Gram-positive bacterium as *Bacillus thuringiensis*. Metagenomic analysis of the infectious masses collected from *Aedes fluviatilis* revealed the presence of gene coverage with identity mainly to uncultured microorganisms and to the 13 most prevalent bacterial genera shown in **Figure 5**. However, this methodology did not allow for a more precise identification of an etiological source closely related to the pathology.



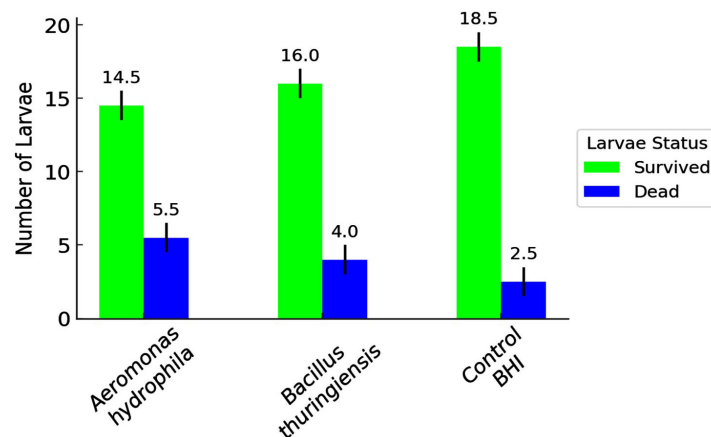
**Figure 5.** Representation of the sequences with the highest coverage according to their closest genetic identity.

### 3.2. Biocontrol Test

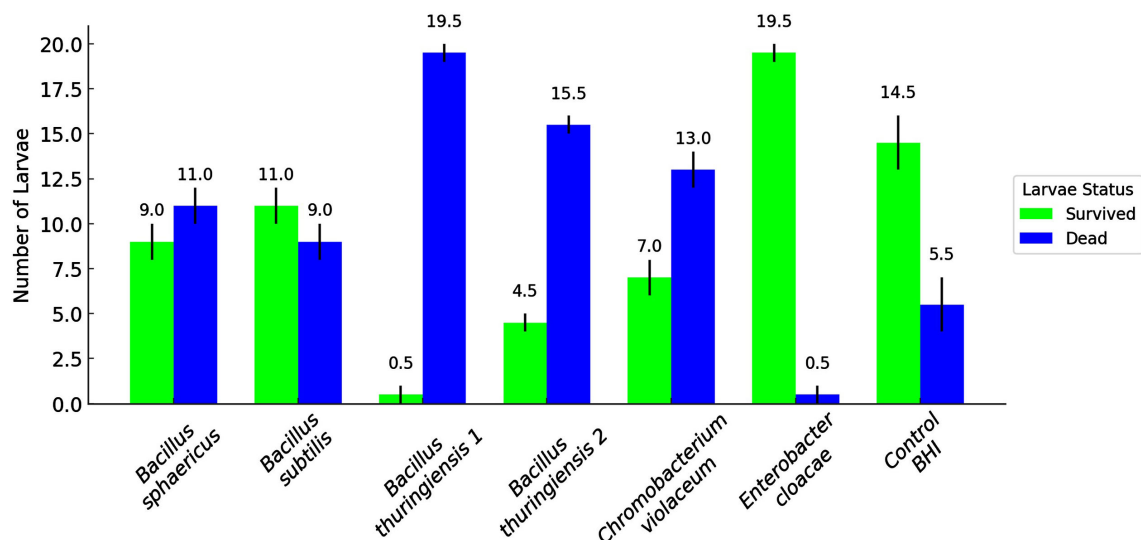
The first experiment showed reduced survival in the containers with microbial treatments of *Aeromonas hydrophila* or *Bacillus thuringiensis*, as indicated in **Figure 6**. After the initial experiments, we did not observe significant inhibitory effects for the two isolates of the infectious masses, even when used together.

Although increased mortality of mosquito larvae by *A. hydrophila* was observed, repeating these experiments multiple times did not demonstrate a good inhibitory activity of the microorganisms isolated from infectious masses in controlling wild *Aedes* mosquitoes. Therefore, we conducted new bioassays to screen for other environmental isolates that are more effective in biological control. We selected six environmental isolates from the bacteriotheque of the Microbiology Laboratory that showed the best results in antilarval screening bioassays. These isolates were identified by MALDI-TOF and sequencing of 16S ribosomal and replicase genes, being 01 *Chromobacterium violaceum*, 01 *Enterobacter cloacae*, 01 *Bacillus cereus*, 01 *Bacillus sphaericus*, and 02 *Bacillus thuringiensis*. The

bioassays with these microorganisms are shown in the **Figure 7**.

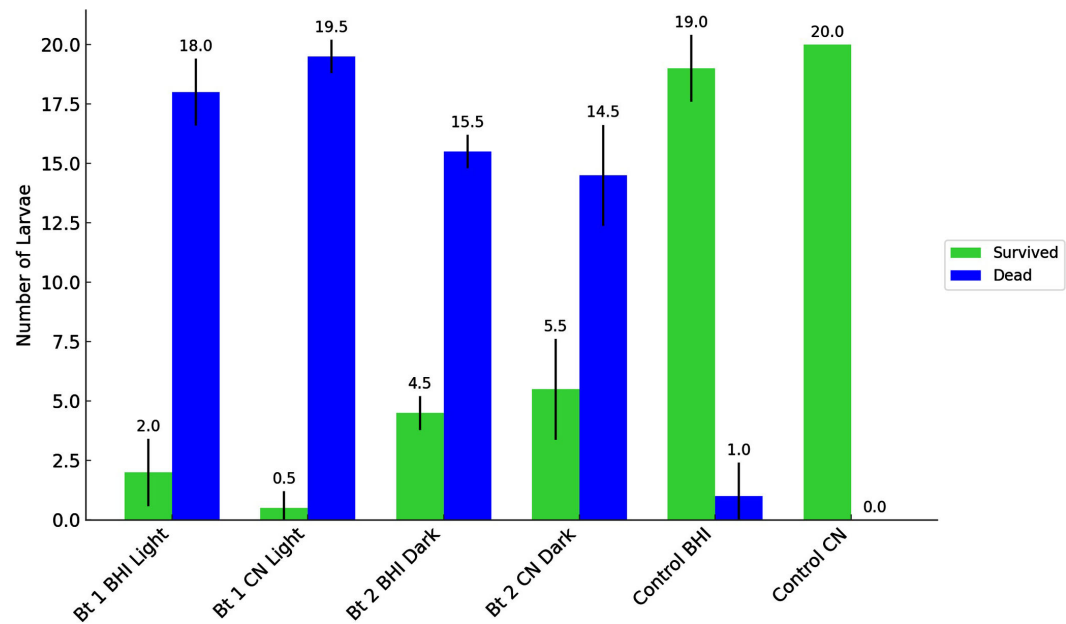


**Figure 6.** Media of microbial treatment using *B. thuringiensis* and *A. hydrophila* isolates grown in BHI for 72 h to biocontrol of *Aedes* mosquito larvae, p-value = 0.333.



**Figure 7.** Media of microbial treatment using environmental strains grown in BHI for 72 h to biocontrol of *Aedes* mosquito larvae. ANOVA Tukey's HSD post-hoc test showed that all treatments except *Enterobacter cloacae* had a significantly different number of surviving and dead larvae than the Control BHI group.

We selected two strains of *B. thuringiensis israelensis* from the bioassays above due to their excellent inhibitory results, environmental safety, and widespread use in biological control. We conducted additional tests using two types of culture media: one with animal nutrients (BHI) and the other of plant origin (Nutrient Broth—NB). We also tested the influence of microbial growth under daylight (environmental illumination) and dark conditions. Nutritional sources did not alter the inhibitory capacity of *B. thuringiensis* cultures (**Figure 8**). However, although not statistically significant, cultures grown under light showed more larval deaths compared to microorganisms cultivated in dark conditions.



**Figure 8.** Bioassays with *Bacillus thuringiensis* (Bt) strains n° 1 and 2, grown in BHI and CN broths, under daylight and dark conditions, for the analysis of inhibitory activity in 2nd instar larvae of *Aedes* mosquitoes.

#### 4. Discussion

Globally, *Aedes aegypti* and *Aedes albopictus* have been the main vectors responsible for the transmission of significant arboviral diseases, such as Zika fever, dengue fever, chikungunya fever, yellow fever, Mayaro fever, and others [15]-[17].

Current control measures aim to eliminate these mosquitoes in different stages. Still, in general, the effectiveness of these interventions has been very low, and epidemics continue to occur in urban centers [2]. The discovery of the Gram-positive soil bacterium *Bacillus thuringiensis*, which produces a toxin highly toxic primarily to dipteran larvae of the *Culicidae* family, with advantages such as not polluting the environment and being non-toxic to animals, has enabled the development of new bioinsecticides that have become a safe alternative [18]-[20].

Regarding the infection of 2nd and 3rd stage larvae of *Aedes fluviatilis*, which exhibited a whitish and rounded mass in the posterior part of the abdomen that overgrew, hindering movement to reach the air interface, sometimes covering the entire respiratory siphon and preventing respiration, it was not possible to reproduce the disease in wild *Aedes* mosquitoes. The disease decimated the colony of *Aedes fluviatilis*, leading us to collect wild *Aedes* eggs for the bioassays. According to Miyazaki *et al.* [21], traps were placed for mosquito oviposition, mostly *Aedes aegypti*, following the points previously studied and established by the Entomology Laboratory. We found no data in the literature reporting a similar disease in *Aedes* spp mosquito larvae. Therefore, we established some hypotheses that might have occurred, either in isolation or concurrently, for the non-reproduction of the disease in wild mosquitoes: 1) The infection may be specie-specific; 2) The infection occurs by non-cultivable microorganisms or through them in the microbial consortium; 3) Use of strains with low or lost virulence due to environmental

factors, especially nutritional factors of microorganisms and larvae; 4) low viability of *A. fluviatilis* colonies.

The microorganisms isolated from aerobic cultures were identified as *Aeromonas hydrophila* and *Bacillus thuringiensis*. The genus *Aeromonas* comprises a group of Gram-negative species that might be found in the mosquito microbiota [22]. *Bacillus thuringiensis*, however, is a well-established environmental microorganism in pest biocontrol, including mosquitoes [20] [23] [24].

Initial bioassays screening with bacteria from the Microbiology Laboratory's bacteriothèque revealed six potential strains, identified as *Chromobacterium violaceum*, *Enterobacter cloacae*, *Bacillus cereus*, *Bacillus sphaericus*, and *Bacillus thuringiensis* var. *israelensis* (n° 1 and n° 2). But, after the repetition of bioassays, the strains with the best inhibitory efficacy against *Aedes* larvae were selected, including two Gram-positive (*Bacillus thuringiensis* n° 1 and n° 2) and one Gram-negative (*Chromobacterium violaceum*). These three strains were selected for future research due to their safety for human and animal health and environmental friendliness. Additionally, Bti species have been widely employed, especially as entomopathogenic agents in formulations of biocontrol products [24]. In the case of *Bacillus thuringiensis*, there will be a subsequent need to characterize its toxins.

*Chromobacterium vaccinii* has lethal activity against mosquitoes [25]. Burkina Faso *C. violaceum* strain after infection of insecticide-resistant *Anopheles coluzzii* causes reduction of both mosquito blood feeding and fecundity propensities [26]. Recently, a *Chromobacterium* strain from the midgut microbiota of *Aedes aegypti* significantly reduced mosquito susceptibility to *Plasmodium falciparum* and dengue virus infection, compromising vector competence [27]. Other studies also reveal the biocontrol action of *C. violaceum* on mosquito larvae and adult forms [28] [29]. Thus, due to the low virulence of environmental isolates of some *Chromobacterium* spp. and their potential for mosquito biocontrol, future studies should be conducted to better evaluate their biosafety, industrial scalability as a bioproduct, field application feasibility, field efficacy, and other interactions, including with insects.

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## Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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