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Vishnu Suresh
Department of Dairy Microbiology, 2018 Batch M. Tech. Student, College of Dairy Science and Technology (Renamed as Verghese Kurien Institute of Dairy and Food Technology), Kerala Veterinary and Animal Science University, Thrissur, Kerala, India

Ligimol James
Department of Dairy Microbiology, Verghese Kurien Institute of Dairy and Food Technology, Kerala Veterinary and Animal Science University Mannuthy, Thrissur, Kerala, India

Dr. AK Beena
Department of Dairy Microbiology, Verghese Kurien Institute of Dairy and Food Technology, Kerala Veterinary and Animal Science University Mannuthy, Thrissur, Kerala, India

Dr. Aparna Sudhakaran V
Department of Dairy Microbiology, Verghese Kurien Institute of Dairy and Food Technology, Kerala Veterinary and Animal Science University Mannuthy, Thrissur, Kerala, India

Dr. Divya MP
Department of Dairy Chemistry, Verghese Kurien Institute of Dairy and Food Technology, Kerala Veterinary and Animal Science University Mannuthy, Thrissur, Kerala, India

Amrutha TA
Department of Dairy Microbiology, Verghese Kurien Institute of Dairy and Food Technology, Kerala Veterinary and Animal Science University Mannuthy, Thrissur, Kerala, India

Corresponding Author:
Vishnu Suresh
Department of Dairy Microbiology, 2018 Batch M. Tech. Student, College of Dairy Science and Technology (Renamed as Verghese Kurien Institute of Dairy and Food Technology), Kerala Veterinary and Animal Science University, Thrissur, Kerala, India

Assessment of motility, exopolysaccharide production, biofilm formation of bacteria isolated from pasteurized cow milk and impact of simulated Cleaning-In-Place (CIP) procedure on the produced biofilms

Vishnu Suresh, Ligimol James, AK Beena, Aparna Sudhakaran V, Divya MP and Amrutha TA

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Abstract

Biofilms which are aggregates of bacteria protected by layers of protective polysaccharide or polymers represent a challenge of great concern for the dairy sector. Detachment of bacteria from these biofilms leads to contamination and complications such as increased corrosion, reduced heat transfer efficiency, and microbiological hazards, all of which can translate into significant financial losses for dairy producers. Contamination of the processed products can harm the quality of the product and pose serious hazards to the consumers.

The reported study assessed the biofilm formation ability, motility of four bacterial cultures namely, *Aeromonas caviae* DMV01, *Moraxella osloensis* DMV03, *Carnobacterium maltaromaticum* DMV05, and *Carnobacterium maltaromaticum* DMV06 isolated from market samples of pasteurized cow milk and the effectiveness of Cleaning-In-Place (CIP) processes in removal of biofilm through simulated studies. Only one culture, Aeromonas *caviae* DMV01 was found to be motile. This organism exhibited swarming motility too. Among the tested strains, only *A. caviae* DMV01 and one of the *C. maltaromaticum* strains, DMV05 were able to form biofilms with the former being more robust than the latter in biofilm formation. On assessing the effectiveness of CIP in removing the biofilm formed by *A. caviae* DMV01, 100% and 82.7% reduction in *A. caviae* count was observed on using CIP solutions at one per cent and 0.5% level, respectively. Observations of the current study underscore the strain specificity in biofilm formation potential of bacteria and highlight the essentiality of conducting industry/establishment wise studies to formulate and adopt tailored cleaning regimes targeting the biofilm forming microflora prevalent in that particular industry/establishment, rather than adopting a universal one.

Keywords: Biofilms, dairy sector, bacterial contamination, cleaning-in-place (CIP)

Introduction

Biofilms are multicellular communities of bacteria living in and multiplying in a matrix made of polysaccharides or other polymers of bacterial origin (Wang *et al*., 2021) [30]. They are recognized as serious concerns in dairy industry as the pathogenic and other microorganisms may detach from the biofilm compromising the safety and quality of the processed milk products (Yan *et al.*, 2023) [31]. Additionally, biofilms are found to create serious problems like increase in corrosion rates on metal surfaces, decrease in heat transfer efficiency during heat treatments, reduction in flow in pipes, increase in frictional resistance of fluids, and reduction in the microbiological quality of products, eventually leading to financial losses (Cherif-Antar *et al*., 2016) [5]. Formation of biofilms in milk storage tanks, transport pipes and milk filters is identified as the main source of food risk in the dairy industry (Kutuk and Temiz, 2022) [16]. Bacteria present in milk are reported to form biofilms in the canalization of dairy industry (Marchand *et al*., 2012) [18]. In addition to the availability of nutrients, microbial cell properties like cellular membrane components, appendages such as pili, flagella and bacteria-secreted extracellular polymeric substance (EPS) are found to contribute towards biofilm formation (Rodrigues *et al*., 2024) [25].

Biofilm formation by bacteria capable of surviving pasteurization and remain viable in milk products, constitutes a major liability for the dairy industry as the possibilities of exposing consumers to danger is very high (Cherif-Antar *et al*., 2016) [5]. As the biofilms can protect the forming microorganisms from high temperatures and chemical compounds applied during sanitization procedures like cleaning in place (CIP) processes which are designed to maintain a clean and hygienic environment (Bayoumi *et al*.. 2012) [3], they pose a great challenge to the dairy industry. Taking all these aspects into consideration the reported study aimed to assess the biofilm formation potential and motility of four bacterial strains isolated from market samples of pasteurized milk and the effectiveness of simulated Cleaning-In-Place (CIP) procedure using different concentrations of solutions on the biofilm produced by these organisms on stainless steel surfaces.

Materials and Methods

Four bacterial strains namely *Aeromonas caviae* DMV01 (MT071634), *Moraxella osloensis* DMV03 (MT158663), *Carnobacterium maltaromaticum* DMV05 (MT158664) and *Carnobacterium maltaromaticum* DMV06 (MT158665) isolated from market samples of pasteurized milk at Department of Dairy Microbiology, Verghese Kurien Institute of Dairy and Food Technology, Mannuthy were used in this study. The 16S rRNA gene sequences are available in GenBank under the given accession numbers. For the experiments, the isolates stored in nutrient broth and nutrient agar plates at 4 °C were activated by inoculation into nutrient broth and subsequent incubation at their optimum growth temperatures (37 °C for DMV 01, DMV03 and 20 °C for DMV05, DMV06).

Assessment of swimming motility using Semi Solid Agar – Triphenyl Tetrazolium Chloride Method

Motility of the cultures was tested as per Ball and Sellers (1966) [2] using the dye 2,3,5- triphenyl tetrazolium chloride (TTC). In brief, broth cultures of the isolates were stabbed into each of the TTC incorporated 5ml soft nutrient agar (with 0.8 per cent agar) and incubated at respective temperatures for 24 h. Spreading of growth from the line of stab was considered as indicative of the motile nature of the isolate.

Assessment of Swarming Motility of selected cultures

Swarming motility agar was prepared using one per cent tryptone, 0.5 percent sodium chloride, and 0.25 percent agar (HiMedia). A portion from the colony of the culture developed on a streak plate incubated at 37 °C for 24 h was transferred with a sterile toothpick to the center of the agar plate, and the plates were incubated face up at 37 °C for 16 to 24 h. Migration of the bacteria from the point of inoculation to the periphery of the plate creating a zone-like appearance was considered indicative of its swarming motility (Rabaan *et al*., 2001) [23]. The swarming motility of the isolate was captured using a Trinocular microscope (Motic).

Qualitative detection of EPS production on Congo red agar

Preliminary assessment of biofilm formation potential of the cultures was done using Congo Red Agar. The cultures were streaked on Congo red agar (CRA) and were incubated at their optimum temperatures for 24 h (Freeman *et al*., 1989) [9] and development of black colonies was considered as indicative of their biofilm formation potential.

Biofilm formation on glass surfaces with the test tube method

The tube method as described by Christensen *et al.* (1982) [6] was performed by inoculating a loopful of culture (turbidity equivalent to 0.5 McFarland solution) into 10 ml trypticase soy broth with one per cent glucose. The tubes were incubated at 37 °C for 24 h. After that, the tubes were decanted, washed with phosphate buffer saline of pH 7.3 and dried. These tubes were then stained with crystal violet and rinsed with deionized water to remove the excess stain. Tubes were dried in the inverted position and observed for the appearance of a line of violet colour on the sides and bottom of the test tubes indicating their biofilm formation potential.

Scanning Electron Microscopy of biofilms formed on glass slips

Sterilized 10 ml trypticase soy broth (TSB, HiMedia Laboratories Pvt. Ltd., Mumbai) tubes were inoculated with 100 µl (10^6 CFU/ml) of biofilm forming *A. caviae* DMV 01 (turbidity equivalent to 0.5 McFarland solution) and non-biofilm forming *M. osloensis* DMV03 cultures. Cover glass slips of less than one cm diameter were inserted into the inoculated test tubes and the tubes were incubated at 37 °C for 32 h. After incubation, the broth was drained out from the test tubes, and the glass slips were washed three times with sterile normal saline and air dried at room temperature (Korres *et al.,* 2013) [15]. Glass slips were mounted on aluminum stubs, sputter coated with gold and observed using Tescan Vega 3 (Czech Republic) scanning electron microscope operated at 5kV and different magnifications.

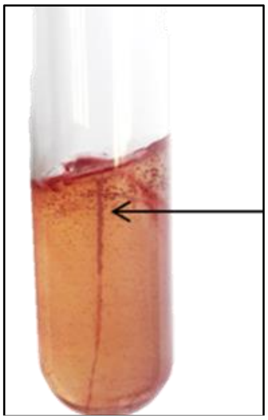
Assessment of effect of Cleaning-In-Place (CIP) Procedure on biofilms formed by *A. caviae* DMV01 on stainless steel (SS) strips

Four stainless steel (SS) strips each of size 1 cm×2.5 cm were dipped in four 10 ml TSB tubes inoculated with *A. caviae* DMV01 culture at two per cent level (Turbidity equivalent to 0.5 McFarland solution) and incubated for 72 h at 37 °C for allowing the formation of biofilm, if any (Angeles-Morales *et al.*, 2012) [1]. After incubation the broth was decanted and the strips were collected using a sterile forceps. To obtain the initial count, one face of one of the strips was swabbed using a cotton swab, the swab was then dipped and mixed thoroughly in 10 ml of 0.1 per cent peptone water (HiMedia Laboratories Pvt. Ltd., Mumbai). Appropriate dilutions were made from this and plated using plate count agar (PCA) and incubated at 37 °C for 24 h. To simulate the industrial CIP conditions in laboratory, a standard CIP regimen of 5 min cold water rinse, a 10 min wash with NaOH at 65 °C, a 2 min cold water rinse, a 10 min wash with nitric acid (HNO_3) at 65 °C, and a final 5 min cold water rinse was selected (Dufour *et al*., 2004) [7]. Two of the remaining three strips were used for assessing the effect of two different concentrations (0.5 and 1 per cent) of CIP solutions (NaOH and HNO_3) (Bremer *et al.*, 2006) [6]. For this, each of the strips were placed into 250 ml bottles, the bottles were filled manually with 100 ml each of the solutions mentioned in the CIP regimen in a sequential manner and simultaneously incubated in a shaking incubator at 250 rpm for the time period specified for each of the solutions (Ostrov *et al.*, 2019) [21]. The fourth strip was also treated in the same way except that the CIP solutions were replaced with sterile water in order to serve as the control. After the CIP procedure, swabs were taken from one side of each of these three SS strips, dipped and mixed well in 10 ml of 0.1 per cent sterile peptone water. Appropriate dilutions were made from this and pour plated using PCA and incubated at 37°C for 48 h. The colonies developed were counted and the result was expressed as CFU/cm^2 of the stainless-steel strip.

Results

Assessment of motility

On assessing the swimming motility of the cultures using semi solid agar *A. caviae* DMV01 alone was found growing away from the line of stab indicating its motile nature (Fig. 1). *A. caviae* DMV01 also exhibited surface movement on swarm agar (Fig.2). The consistent growth circle formed on the swarm agar Petri dishes clearly indicated the swarming motility of *A. caviae* DMV01. These observations are in agreement with the report of exhibition of both swimming and swarming motilities by a diarrhea-associated isolate, *A. caviae Sch3N* (Lowry *et al.*, 2014) [17]. Exhibition of both swimming and swarming motilities by *A. caviae* DMV01 is of special relevance as swarming motility which has been linked to the formation of biofilms (Kirov *et al.*, 2002) [14] is exhibited by only 60% of mesophilic *Aeromonas* strains (Kirov *et al.*, 2004) [12].



Growth of *A. caviae* DMV01 away from the stab line

Fig 1: Semi solid agar tube containing triphenyl tetrazolium chloride exhibiting swimming motility of *A. caviae* DMV01

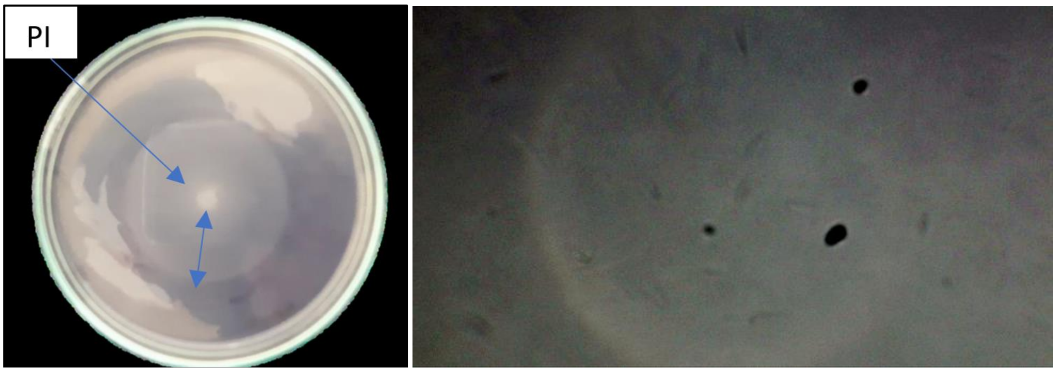


Fig 2: Analysis of swarming motility of *A. caviae* DMV01 a. Growth around the axis from the point of inoculation (PI) on swarm motility agar b. Swarming motility of *A. caviae* DMV 01 under microscopic

EPS Production and Biofilm formation

Among the cultures assessed, *A. caviae* DMV01 and *C. maltaromaticum* DMV05 formed black discolouration on Congo red agar indicating their ability to form EPS (Fig.3). Production of exopolysaccharide by a strain of *A. salmonicida* reported by Garrote *et al*. (1992) [10] and presence of loci potentially involved in the synthesis of exopolysaccharides in the genome of *C. maltaromaticum* LMA 28 reported by Rahman *et al*. (2014) [24] are supportive of the observations of the current study. The test tubes inoculated with *A. caviae* DMV01 and *C. maltaromaticum* DMV05 retained violet colour on their sides and bottom, indicating the biofilm formation potential of these two cultures with *A. caviae* DMV01 exhibiting stronger biofilm formation than the other (Fig.4). Production of EPS and biofilm by only one of the strains of the two *Carnobacterium* strains clearly indicates the strain specificity in these attributes.

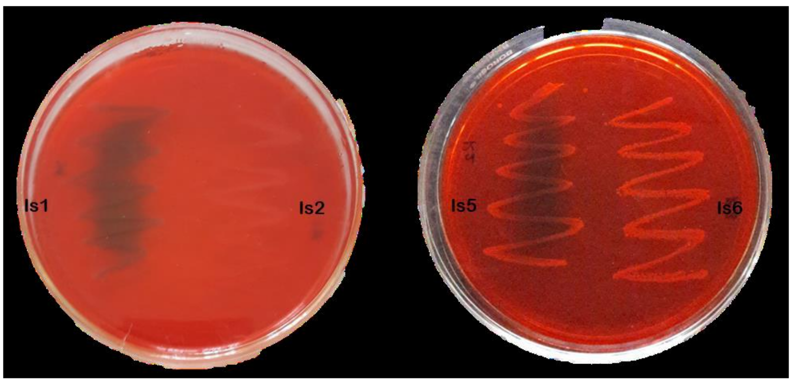


Fig 3: Black discolouration on colonies formed by *A. caviae* DMV01 (Is1) and *M. osloensis* DMV03 (Is2) on Congo red agar plates indicating EPS production

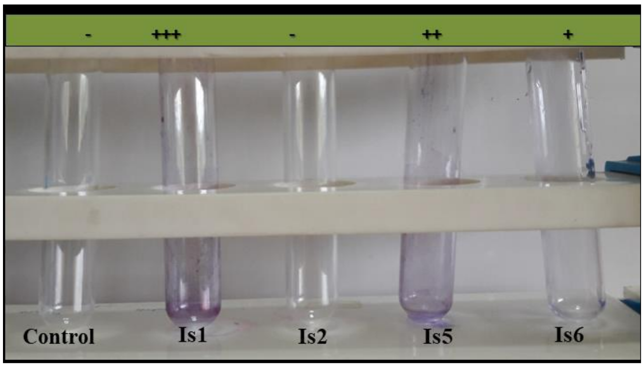


Fig 4: Retention of crystal violet on test tubes indicating biofilm formation potential of Is1- *A. caviae* DMV01 (Is1) and *C. maltaromaticum* DMV05 (Is 5). Is2- *M. osloensis* DMV03, Is6-*C, maltaromaticum* DMV06

High vacuum scanning electron microscopy at a magnification of 5000x revealed that *A. caviae* DMV01 is capable of forming biofilms on the glass surface (Fig.5). Numerous cells of *A. caviae* DMV01 appeared as a thick layer on the glass surface, whereas only a few cells were present in the case of *M. osloensis* (Fig. 5 A, B). At higher magnification, the presence of some extracellular protrusions and slime like substances were visible in between the rod-shaped cells (Fig. 5C).

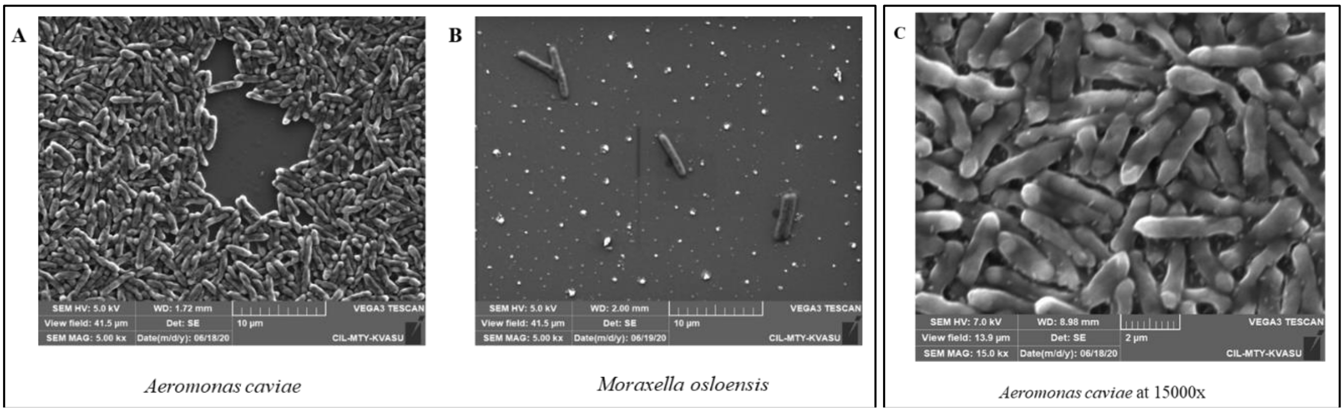


Fig 5: Scanning electron micrographs of biofilm formation on glass surface by (A) *A. caviae* DMV01(magnification 5000X) (B) *M. osloensis* DMV03 (magnification 5000X) (C) *A. caviae* DMV01 (magnification 15000x)

Agreeing with the reports of important roles of bacterial motility in biofilm formation (Merritt *et al*., 2007) [19], *A. caviae* DMV01, the only motile culture in this study was identified as a biofilm producer. Ability of members of the genera *Aeromonas* to produce biofilm is well reported (Talagrand-Reboul *et al*., 2017; Seike *et al*., 2021; El-Hossary *et al*., 2023) [29, 26, 8]. The critical role played by polar flagellum of *Aeromonas* spp. in biofilm formation and colonization of surfaces was well demonstrated for *A. caviae* strain Sch3 by Kirov *et al*. (2004) [12]. From the dairy industry point of view, biofilm forming microorganisms are of high public health and economic relevance as these microorganisms can get easily established in milk storage tanks, processing lines resulting in contamination of processed products. Such contaminated products can also act as potential carriers of biofilm detached microorganisms, which is of special relevance in the case of potentially pathogenic strains.

Resistance to Cleaning-in-place (CIP) Procedure of *A. caviae* DMV01 biofilm

Despite its biofilm forming potential *A. caviae* DMV01 was found to be highly sensitive to the CIP regimen to the extent that use of CIP solutions at one per cent level resulted in complete removal of *A. caviae* DMV01 from the biofilm formed on the stainless steel strips (Table 1). A reduction of 3.2 log CFU/cm^2 indicating 82.7% reduction in *A. caviae* DMV01 count compared to the initial count was observed on using the CIP solutions at 0.5 per cent level. The biofilm removal effect of a cleaning agent includes both the mechanical effect of the liquid circulation and the chemo-biological effect from the active components, present in the agent (Ostrov *et al*., 2016) [21]. As adoption of CIP regimen with sterile water alone could reduce the viable *A. caviae* DMV01 population to 48.6% of the initial population, it could be inferred that the biofilm formed was highly susceptible to the mechanical effect of cleaning. The comparatively higher reduction in the count observed on using chemicals shows the susceptibility of *A. caviae* DMV01 cells to both the mechanical and chemo-biological effects of the employed solutions. The observation of partial/complete removal of *A. caviae* DMV01 cells from the biofilms formed on stainless steel strips on treatment with 0.5 and one per cent CIP solutions used in a laboratory level simulation is comparable to the report of Parkar *et al.* (2004) [22]. As in the current study, these authors also reported a decrease in the effectiveness of standard CIP regimen to remove the biofilms formed on stainless steel coupons by *Bacillus flavothermus,* on decreasing the strength of the solutions. Complete removal of the cells from the surface on using one per cent CIP solutions in combination with the turbulence offered by 250 rpm agitation indicates that the *A. caviae* DMV01 is not resistant to the cleaning in procedure adopted in this study. Seike *et* *al*. (2021) [26] reported the involvement of outer membrane vesicles released from *Aeromonas* in its biofilm formation and that the extracellular DNA molecules and proteins existing in extracellular matrix may play an important role in maintaining the *Aeromonas* biofilm. So, use of NaOH, an efficient agent against proteins and carbohydrates might have contributed towards the added efficiency obtained on using the cleaning agents. Additionally, the very short age of the tested biofilm could also have contributed to its vulnerability to the CIP simulation adopted. In contrast, resistance to cleaning procedures is reported for many biofilm formers. Jahid and Ha (2014) [11] observed higher resistance to disinfectants by *Aeromonas hydrophila* biofilms than planktonic cells. Several studies have reported the survival of *Bacillus cereus* and its biofilms even after sanitization (Zhou *et al.*, 2008; Shi and Zhu, 2009; Ostrov *et al.*, 2016; Silva *et al.*, 2018) [32, 28, 21, 27].

Table 1: Count (Log CFU/cm^2) of biofilm forming *Aeromonas caviae* DMV01 on stainless steel after different treatments

	Prior to CIP	Control (CIP with sterile water)	Treatment 1 (CIP regimen with 0.5% CIP solutions)	Treatment 2 (CIP regimen with 1% CIP solutions)
Log CFU/cm^2	3.87±0.06	1.99±0.04	0.67±0.14	0
% Reduction compares to the initial count		48.6	82.7	100

Values shown are Mean ± S.E. of three experiments

Conclusion

In the present study four bacterial cultures isolated from pasteurized cow milk were assessed for their motility, exopolysaccharide biofilm production. Out of the tested cultures, *A. caviae* DMV01 was found to exhibit both swimming and swarming motility. This culture and one of the strains of *C. maltaromaticum,* DMV05 were found to be exopolysaccharide and biofilm producers. Among these two cultures, *A. caviae* DMV01 was found to be a stronger biofilm producer and found to be susceptible to standard CIP procedures with the extent of susceptibility varying with the concentration of the CIP solutions or the procedure used. Findings of this study emphasize strain specificity in biofilm production and the need for developing industry specific, tailored cleaning protocols against biofilm-related contamination to ensure the safety and integrity of dairy products. However, *in situ* validation of the findings of this study in complex dairy systems, like pipes and a variety of surfaces, are required to develop advance strategies for managing biofilms in the dairy industry.

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