



# Microbiological analysis of raw milk unveiled the presence of a dairy contaminant, *Corynebacterium lipophiloflavum*

Shih-Keng Loong<sup>1</sup>, Hai-Yen Lee<sup>1</sup>, Jing-Jing Khoo<sup>1</sup>, Fang-Shiang Lim<sup>1,2</sup>, Siti-Noraisah Ahmad-Nasrah<sup>2</sup>, Adzzie-Shazleen Azman<sup>1</sup>, Chubashini Suntharalingam<sup>3</sup>, Chandrawathani Panchadcharam<sup>4</sup>, Sazaly AbuBakar<sup>1,2\*</sup>

<sup>1</sup>Tropical Infectious Diseases Research and Education Centre, Faculty of Medicine, University of Malaya, 50603 Kuala Lumpur, Malaysia, <sup>2</sup>Department of Medical Microbiology, Faculty of Medicine, University of Malaya, 50603 Kuala Lumpur, Malaysia, <sup>3</sup>Economics and Social Science Research Centre, Malaysian Agricultural Research and Development Institute (MARDI), 43400 Serdang, Malaysia, <sup>4</sup>Department of Veterinary Services, Laboratory Diagnostics Division, Wisma Tani, Federal Government Administrative Centre, 62630 Putrajaya, Malaysia

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## ABSTRACT

Dairy farming occupied a distinct position in agriculture since milk can be harvested every day, providing a regular source of income to the farmers. Development of the Malaysian dairy farming industry was marred by poor farm hygiene practices, leading to the proliferation of dairy-spoilage bacteria, affecting milk quality. In this study, we report the isolation and characterization of a rare *Corynebacterium* species from raw milk after the implementation of improved farm hygiene practices. All milking equipment, farm worker's hands and the cow's udders and teats were washed with detergent and wiped dry with clean towels before milk sample collection. Collected foremilk samples from mastitis-free cows were inoculated onto Petrifilm™ and cultured colonies were plated onto nutrient agar. Biochemical and molecular tests were performed for the identification of peculiar bacterial isolates. A unique yellow-pigmented bacteria isolate was recovered from the milk of a healthy cow after the adoption of improved farm hygiene practices. Phenotypic and genotypic characterization confirmed the milk isolate as *Corynebacterium lipophiloflavum*. This is the first description of *C. lipophiloflavum* in cow's milk and could possibly imply the influence of bovine flora in dairy contamination. The findings highlight the increasing spectrum of *Corynebacterium* species with potential adverse impact to the dairy industry. It is recommended to screen for *C. lipophiloflavum* in all milk processing facility to ensure that milk is safe for consumption and its products prepared to the highest quality and safety standards.

## 1. INTRODUCTION

Milk is a food commodity valued for its nutrition besides providing regular income for the dairy farmers [1]. In Malaysia, high local demand for dairy products resulted in the country having to rely substantially on imports [2]. The development of the local dairy production capacity began since 1974 when the Malaysian government initiated the National Dairy Development Program to reduce dependency on milk imports [2]. However, up to date, the local dairy industry still is unable to meet the demand of the growing population. Efforts are also made by local extension personnel on ensuring that while milk production capacity increases, milk quality is also maintained so as to ensure that food safety is not compromised [2]. The decrease in milk quality can be attributed to the presence of microorganisms and problems

with hygiene practices [3]. Improvement in farm hygiene practices has been demonstrated to reduce bacterial count and mold in raw milk [4], elevating milk quality by 2.4 times reduction of risk exposure to milk pathogens and its potential toxins. Members of the genus *Corynebacterium* include species that occasionally cause infection in humans and some species having been recovered solely from animals, the environment, food, water, and synthetic materials [5]. *C. ulcerans*, for instance, was thought to cause disease in farm workers after contact with contaminated milk or farm animals [5]. Here, we performed a microbiological analysis on raw milk after the implementation of improved farm hygiene practices to assess the impact of the initiatives. We report recovery of a rare *Corynebacterium* species from raw milk after the implementation of improved farm hygiene practices.

## 2. MATERIALS AND METHODS

Before the start of milking, the udders and teats of healthy dairy cows and the hands of farm workers were rinsed with 1.0% diversol and wiped dry with clean towels. Milking equipments were washed with sanitizing solution comprising of 5.4 g iodophor diluted in 10 L of

### \*Corresponding Author

Sazaly Abu Bakar,  
Tropical Infectious Diseases Research and Education Centre,  
Faculty of Medicine, University of Malaya,  
50603 Kuala Lumpur, Malaysia. Phone: +603 79676670.  
Email: sazaly@um.edu.my

**Table 1:** Biochemical and antimicrobial susceptibility profiles of *Corynebacterium lipophiloflavum* isolated from raw milk

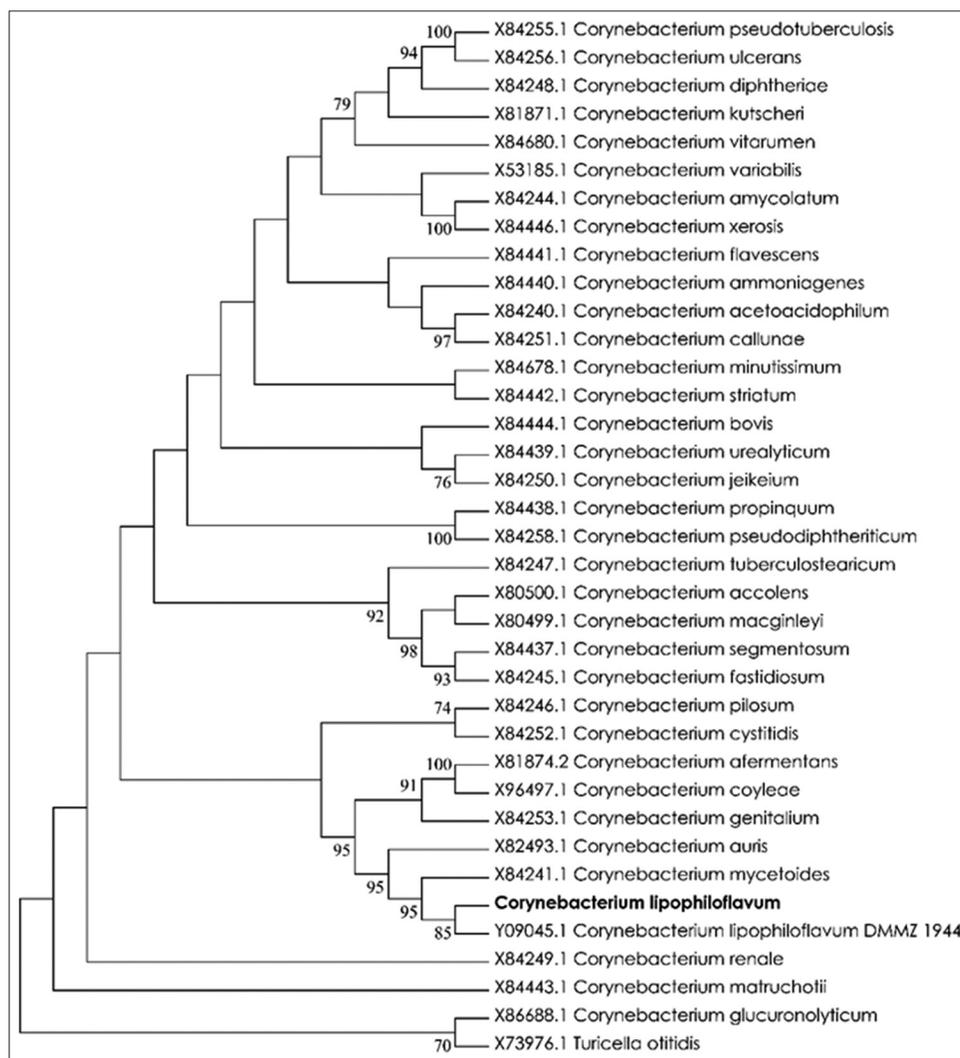
Biochemical test	Result
Urea hydrolysis	+
Glucose fermentation	-
Maltose fermentation	-
Sucrose fermentation	-
Antimicrobial susceptibility	Result (MIC, µg/mL)
Cefotaxime	S (1.0)
Ceftriaxone	S (1.0)
Ciprofloxacin	S (0.5)
Erythromycin	S (0.5)
Gentamicin	S (0.5)
Meropenem	S (0.12)
Penicillin	S (0.12)
Tetracycline	S (1.0)
Vancomycin	S (1.0)

+: Positive for the biochemical test, -: Negative for the biochemical test, S: Susceptible to the antibiotic, MIC: Minimum inhibitory concentration

water and rinsed with clean water before the milking process. Foremilk specimen was collected directly from the teat after discarding the first milk. All milk specimens were collected from mastitis-free cows and inoculated onto the respective Petrifilm™ (*Escherichia coli* and coliform, yeast and mold, total aerobic count, *Staphylococcus aureus*, and *Enterobacteriaceae*) (3M Corporation, St. Paul, MN, United States) and enumerated [4]. Colonies on the films were then plated onto nutrient agar and incubated under aerobic conditions at 37°C for 24–48 h.

### 3. RESULTS AND DISCUSSION

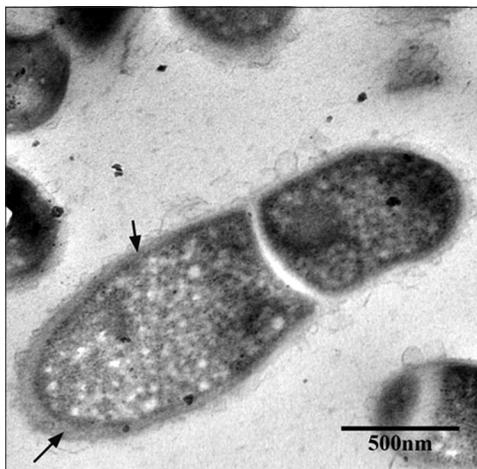
One colony originating from the *Enterobacteriaceae* film grew unique yellow-pigmented colonies between the sizes of 0.5 and 1.0 mm after 48 h incubation. Molecular identification using 16S rDNA sequencing [6] showed that the isolate shared 99% sequence similarity to *Corynebacterium lipophiloflavum* DMMZ 1944 (accession no. Y09045) [Figure. 1]. Due to the close 16S rDNA similarity between different *Corynebacterium* strains, selected differentiating biochemical tests were performed. The *Corynebacterium* isolate was able to hydrolyze urea but could not ferment glucose, maltose, and sucrose [Table 1] similar to *C. lipophiloflavum* [7]. The exhibited biochemical



**Figure 1:** Neighbor-joining tree based on 16S rDNA gene of representative *Corynebacterium* species. Their respective accession numbers are listed before the species names. The *Corynebacterium lipophiloflavum* isolate in this study is indicated in bold. Number at nodes indicates bootstrap values (%) for 1000 replicates.

test results were different to those displayed by its closest phylogenetic relative, *C. mycetoides* [8] [Figure. 1], as such corroborating the 16S rDNA sequencing results, confirming the isolate's identity as *C. lipophiloflavum*. Antimicrobial susceptibility assays performed according to the guidelines by the Clinical and Laboratory Standards Institute [9] demonstrated that the *C. lipophiloflavum* isolate was sensitive to cefotaxime, ceftriaxone, ciprofloxacin, erythromycin, gentamicin, meropenem, penicillin, tetracycline, and vancomycin [Table 1]. The minimum inhibitory concentration assays were repeated twice to verify the results. Visual inspection of the bacterial isolate after incubation in a 96-well plate at 37°C for 48 h [10] noted that the *C. lipophiloflavum* isolate did not produce biofilm. Morphological characteristics of the bacterium unveiled by transmission electron microscopy [11] displayed structures similar to *C. phoceense* [12] and *C. lactis* [13] [Figure. 2]. The bacterium showed characteristic club-shaped cell and the presence of an external lipid layer.

Even though other dairy-spoilage *Corynebacterium* species have been found in raw milk [3], this is the description of the first *C. lipophiloflavum* milk isolate. It was doubtful that *C. lipophiloflavum* was derived from the environment since the sanitization protocols have been proven to be effective in reducing bacterial counts [4]. This bacterium first described from a woman with bacterial vaginosis [7], could probably resist the high hydrogen peroxide concentration environment of the vagina, akin to *C. aurimucosum* [5], and, hence, could possibly be part of the cow's vaginal flora too. There is strong possibility that *C. lipophiloflavum* was transferred from the mother to the calf during birth, comparable to the transfer of vaginal flora from mother to child during delivery [14]. This suggestion was substantiated by the antimicrobial susceptibility profile of the *C. lipophiloflavum* isolate, indicating that it may have emerged from an antibiotic-free environment [15]. Recovery of *C. lipophiloflavum* even after sanitization can be explained by the transfer of endogenous bacteria during milking, which resembled the human milk bacteria colonization of breastfed neonates [16]. Although the *C. lipophiloflavum* isolate did not produce biofilm, it may switch from the dormant state to biofilm producer [17] on leaving the normal flora, as a result of selection and environmental pressures, further suggesting the undesirable influence of corynebacteria in dairy contamination. While *C. lactis* may seem harmless when it was first isolated from the cow's milk, recent studies have shown that it was also found in ticks and can cause infection



**Figure 2:** Morphological characteristics of *Corynebacterium lipophiloflavum* as displayed by transmission electron microscopy at 16,300 times magnification. Arrows show the presence of an external lipid layer.

in companion animals [13]. Hence, treating *C. lipophiloflavum* as a harmless normal flora of the cow could have disastrous aftermath to the entire dairy supply chain. Besides, the finding of *C. lipophiloflavum* in the milk carries significant interest for the dairy industry. It may induce milk spoilage by expressing lipolytic and proteolytic enzymes resembling *C. variabilis* [18], besides potentially causing mastitis akin to *C. bovis* [19]. Furthermore, there are risks to the human health since *C. lipophiloflavum* possesses the ability to cause human infection [7].

#### 4. CONCLUSION

Taken together, the findings highlight the increasing spectrum of *Corynebacterium* species with potential adverse impact to the dairy industry. Previous milk-associated *Corynebacterium* strains identified mostly to the genus level could perhaps be *C. lipophiloflavum* if further characterization was performed. It is recommended to screen for *C. lipophiloflavum* in all milk processing facility to ensure that milk is safe for consumption and its products prepared to the highest quality and safety standards.

#### 5. CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

#### 6. ACKNOWLEDGMENTS

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