Original Article

Exploring the Safety and Hygiene of Enteral Tube Feedings by 16S rRNA Based Sequencing: A Risk Factor in Healthful Nutrition

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Abstract

The enteral tube feeding (ETF) delivery system comprises a key therapeutic tool for preventing the development of malnutrition in hospitalized patients. The microbial composition of ETFs fulfills a crucial role in patient's health, particularly for those who are immunocompromised. The present study aimed to assess the bacterial quality of handmade ETF samples collected from the Imam Reza Hospital of Tabriz, Iran. In total, 45 samples were collected from the ETF preparation kitchen. The bacterial compositions of the samples (counts of total mesophilic bacteria (TMB), *Enterobacter* spp., and *Pseudomonas* spp.) were examined by common microbiological methods. The molecular characterization of the isolated strains was performed via 16S rRNA gene sequencing after PCR amplification. The examination of the ETF samples indicated that no sample had zero TMB count, while 57 % (26/45) of the ETF samples had $\geq 10^4$ colony forming units (CFU) of TMB, $\geq 10^2$ CFU of *Enterobacter* spp., ≥ 10 CFU of *Klebsiella pneumonia*, and $\geq 10^3$ CFU of *Pseudomonas* species. This study reveals that ETFs must achieve a more acceptable level of microbial quality through the implementation of rigorous health principles coupled with continuous monitoring.

Keywords: Enteral Tube Feeding; Microbiological Analysis; 16S rRNA amplification; Polymerase Chain Reaction

Introduction

The enteral tube feeding (ETF) delivery system facilitates the transfer of food into the digestive tract of patients who are unable to eat or must refrain from eating secondary to physical conditions or serious injuries. This method is chiefly utilized in intensive care units (ICU) and neonatal care units, as well as other hospital wards. There is a broad range of raw foodstuff for ETF preparation, with the main difference being in the calories and ingredients incorporated; this ensures compliance with, for example, the divergent nutritional requirements of hospitalized diabetic patients and cancer patients receiving chemotherapy [1]. The food categories in ETF preparation include protein sources, dairy products, fruits, vegetables, and cereals. Once the foodstuffs are blended, the resulting solution is passed through a filter and the liquid part is separated, comprising the ETF. The ingredients of ETFs are prescribed by a clinical nutritionist based on the physical condition and nutritional requirements of the patient. Moreover, commercial ETF powder and liquid are available on the market, with easier and faster preparation. Despite these advantages, research indicates that these instant formulas lack the benefits of handmade solutions, which are non-allergenic and offer higher nutritional value [2]. In the United Kingdom, approximately 300,000 hospitalized patients acquire infectious diseases per year, exerting a major financial load on the economic and health system [3]. The history of ETFs dates back to the nineteenth century; Egeberg was presumably the first who proposed gastrostomy access for the direct administration of medications or nutrients in 1837, while Se˙dillot, in 1845, applied the first gastrostomies for therapeutic purposes [4]. Simultaneous to the development of parenteral nutrition in the 1960s for critically ill patients, ETFs lost their prominence. Nevertheless, ETFs were reinvigorated in the 1990s as a result of studies that showed

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improved patient outcomes with this modality of nourishment relative to its alternatives [5].

To facilitate ETF intake, three common methods are employed including percutaneous endoscopic gastrostomy (PEG), nasogastric gastrostomy (NG), and jejunal gastrostomy (JEJ). Nonetheless, the JEJ and PEG methods pose a high infection risk during both tube insertion and the feeding process [6]. Through ETF contamination, pathogenic and non-pathogenic bacteria duplicate swiftly, which may initiate food poisoning in the consumer with symptoms like nausea, vomiting, fever, diarrhea, and abdominal pain or discomfort. In cases such as cancer chemotherapy, AIDS, severe burns, and other types of immunodeficiency, ETF contamination can eventuate in septicemia, bacteremia, and even death [7]. All of these reasons add to the importance of the ETF's microbial composition.

The improved tolerability of handmade ETFs is probably due to the reduction of symptoms related to the direct ingestion of foods such as retching and gagging. The cost of handmade ETFs could be less than or equal to the commercial type, depending on the foodstuff used [8]. Numerous advantages of ETF have been pointed out in prior investigations, including inexpensive preparation, normalized mealtimes, compliance with special regimes (e.g., vegan; vegetarian), and lack of side effects about direct food consumption like gastric reflux, constipation, food sensitivity or allergy [9].

In hospitals, multiple sources of ETF contamination exist, namely non-hygienic utensils, disregard for hand hygiene, unsuitable upkeep of tubes, and use of polluted water [10, 11]. In light of this situation, some studies have been conducted to improve the ETF quality, and the implementation of the Hazard Analysis and Critical Control Point (HACCP) system in hospital kitchens has been recommended [12]. The present study aimed to evaluate the bacterial quality of ETFs by isolating *Enterobacter* spp. and *Pseudomonas* spp. (two bacterial species highly involved in nosocomial infections) and confirming their presence via 16S rRNA gene amplification.

Materials and Methods

Materials

Bacterial media including peptone water, plate count agar, endo agar, eosin methylene blue (EMB) agar, and cetrimide agar were purchased from Ibresco Co. (Tehran, Iran). The forward and reverse primers and master mix kit were procured from Pishgam Co. (Tehran, Iran).

Enteral tube feeding (ETF) preparation and delivery

A summary of the ETF preparation procedure is depicted in Figure 1. According to the number of patients, the raw materials were transported to the ETF kitchen after delivery from the storeroom (Table 1). Next, the preliminary stages like washing the fruits and vegetables and cooking the chickens and eggs were completed. Then, all of the raw and cooked foodstuffs were conveyed to high-power industrial mixers. Once mixed, the solution was passed through a filter with small pores to achieve the final ETF, which was stored in disposable containers pre-labeled with the patient's name. Next, the ETFs were delivered by the distribution staff to the various hospital wards at 13:00.

Sample preparation and bacterial count

In total, 45 ETF samples were collected; on average, 5 ETF samples were gathered at each sampling time for microbiological tests. For each test, the ETF (10 mL) was dispensed into a zip-pack that had been sterilized under UV lamp for 5 hr, before being transported to the food microbiology laboratory within an icebox. The samples were subsequently diluted through the addition of 90 mL of 0.1 % sterile peptone water, ahead of being homogenized in a stomacher for 1 min at 200 RPM. Serial 10^{-1} to 10^{-6} dilutions of the samples were achieved by adding 1 mL of the homogenized 10^{-1} sample to 9 mL of 0.1 % sterile peptone water followed by mixing using a vortex. Plate count agar was used for the total mesophilic bacteria (TMB); the sample diluents were cultured via the pour plate method and incubated at 37 °C for 48 hr [13]. For *Enterobacter* spp., the sample diluents were cultured by the pour plate method on the endo agar and EMB agar media with incubation at 37 °C for 48 hr [14]. *Pseudomonas* spp. were cultured on cetrimide agar using the spread plate technique and incubation at 25 °C for 48 hr [15]. At the end of the incubation period, the colonies were counted. Furthermore, two colonies were randomly selected from each plate and stored at -24 °C in brain heart infusion (BHI) broth containing 2 % glycerol until further molecular characterization.

DNA extraction and molecular characterization

The bacterial DNA samples were extracted from the isolated strains according to the phenol-chloroform method with slight modifications. The isolated bacterial strains were cultured on nutrient agar for 48 hr to achieve adequate bacterial colonies, which were dissolved in 300 µL of Tris-EDTA (TE) buffer. This was followed by the addition of 30 µL of sodium dodecyl sulfate (SDS), ahead of incubation for 45 min at 40 °C. The mixture was then kept in boiling water for 10 min before 50 µL of phenol/chloroform/isoamyl alcohol (1:25:24, v/v) was added and mixed using a pipette. The aqueous phase was separated and transferred to a fresh microtube, which was supplemented with 100 µL of sodium acetate and 400 µL of isopropanol. The microtubes were then centrifuged for 3 min at 10000 RPM, and the DNA was washed using 1 mL of 75 % ethanol for 30 seconds, before finally being dissolved in 100 µL of TE buffer [16]. The 16S rRNA gene was amplified via the polymerase chain reaction (PCR); universal bacterial primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3') were used [17]. The PCR program was performed via the following steps: (i) first denaturation at 95 ˚C for 5 min; (ii) 40 cycles of denaturation at 95 ˚C for 60 sec, primer annealing at 53 °C for 55 sec, and

extension at 72 °C for 1.25 min; and (iii) final extension at 72 ˚C for 10 min. For 16S rRNA gene sequencing, the PCR product was sent to Macrogen Co. (South Korea). The nucleotide sequence similarity was analyzed by the Chromas software and compared with the registered strains in the EzTaxon Gene Bank database [18] and aligned using Clustal X version 2.0. The evolutionary tree was constructed using the neighbor-joining method [19]. The optimal tree had a total branch length of 4.10483024. The percentage of replicate trees in which the associated taxa are clustered together in the bootstrap test (with 500 replicates) is typically shown next to the tree branches [20]. The evolutionary distances were computed using the Poisson correction method [21] and expressed as the number of amino acid substitutions per site. The analysis involved 17 amino acid sequences. All positions containing gaps and missing data were eliminated. There was a total of 71 positions in the final dataset. The evolutionary analyses were conducted in MEGA7 [22].

Statistical analysis

Statistical analysis was done using SPSS version 23 (SPSS Inc., USA). Wilcoxon Signed Ranks Test was used to compare the TMB count against the US Food and Drug Administration (FDA) standards to evaluate its acceptability. Differences were reported as significant at $p < 0.05$.

Results and Discussion

The neighbor-joining phylogenetic trees of the isolated strains are shown in Figures 2 and 3. Two distinct colony types grew on the EMB agar: creamy, mucoid colonies identified as *Enterobacter asburiae*, and circular, metallic glittery colonies that were characterized as *Enterobacter bugandensis*. On the endo agar, one type of colony was small-sized with a red center, while the other was convex and light pink; these colonies were identified as *Klebsiella pneumoniae* and *Enterobacter cancerogenus*, respectively. On cetrimide agar, only uniform, white, small, mucoid colonies were observed belonging to the *Pseudomonas parafulva* strain. Our results indicated that the bacterial counts were significantly high, and the compliance of the TMB count with the FDA standards was approximately 43 %. Some prior studies on ETF quality have indicated high microbial loads and stressed the importance of observing food safety for hospitalized patients. The results of this survey revealed contamination in 57 % of the ETF samples, which is in line with the findings of Vieira, Santos [23], and Baniardalan, Sabzghabaee [24]. The bacterial counts of the ETF samples and the level of compliance of the TMB with the FDA's microbiological standards are presented in Table 2. Through the exploration of the ETF preparation procedure, the critical points (CPs) of the process (from blending to delivery) that probably led to the high microbial load were identified. Through the employment of preventive procedures, these CPs should be controlled such that the bacterial count of the ETF samples decline and come into compliance with the standards. In other words, a Hazard Analysis and Critical Control Point (HACCP) system are necessary. A summary of the CPs and recommended monitoring actions are cataloged in Table 3. The elevated bacterial counts of the samples indicate the proper conditions for the growth and proliferation of pathogenic bacteria in the ETFs. Other studies have shown the presence of different bacterial strains like *Enterobacter cloacae, Streptococcus* spp. and *Bacillus* spp. in ETFs [25]. The source of contamination can be partially recognized by the microbiological findings of the samples. For example, *P. Parafulva* is a ubiquitous strain that can be found in rice, soil, and water. The most likely causes of such high microbial loads are insufficient cooking of unclean raw foodstuff and cross-contamination from utensils, tools, and food handlers. If adequate sanitization is not ensured, the residual material left overnight on pitchers used for dividing ETFs act as a source of bacterial growth, with instruments like counters and sinks also likely to become contaminated from this source. In this regard, da Silva Santos, Araújo [26] have shown that the presence of food residues on instruments serves as a source of bacterial growth.

Two key factors affecting the ETF quality are microbial quality and usage of suitable ingredients according to the patient's nutritional needs [27]. This highlights the need for much more attention to be directed to ETFs by health authorities. For the bacterial strains that were isolated in this survey, no limits have been defined in the FDA standards [28]; however, we scrutinized the presence of these pathogens given their significance in-hospital infections and the lack of assessment of their prevalence in former studies. *Enterobacter* spp. are considered as important clinical pathogens because of their resistance to antibiotics and the inherent capability of developing resistance to new antimicrobial agents; furthermore, this bacterial genus is recognized as a common source of nosocomial infections [29]. *Pseudomonas* spp. and *Klebsiella pneumonia* is responsible for respiratory and urinary tract infections as well as pneumonia-related to mechanical ventilation [30]. In this study, confirmation of the isolated bacterial strains through 16S rRNA gene amplification confirmed the accurate identification of the strains since this technique is one of the most formidable methods in the detection of bacterial species.

Conclusion

The present study elucidated the significance of utilizing a high standard of hygiene practices combined with consistent monitoring during the ETF preparation procedure. In this study, the level of compliance with the microbial standards was determined to be 43 %, meaning that minimizing the risk of infection through ETF contamination is vital for the preservation of patient health. In conclusion, considering the growing use of ETF nourishment due to its natural components and fewer side effects (e.g., allergies; diarrhea) compared with total parenteral nutrition, it is necessary to adopt standards that guarantee the safety of ETFs in hospital preparation units all around the globe. Future studies should be conducted for detecting the presence of other pathogenic microorganisms and identifying the effects of contaminated ETFs on immunocompromised patients.

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Conflicts of Interest

The authors indicated that they have no conflicts of interest.

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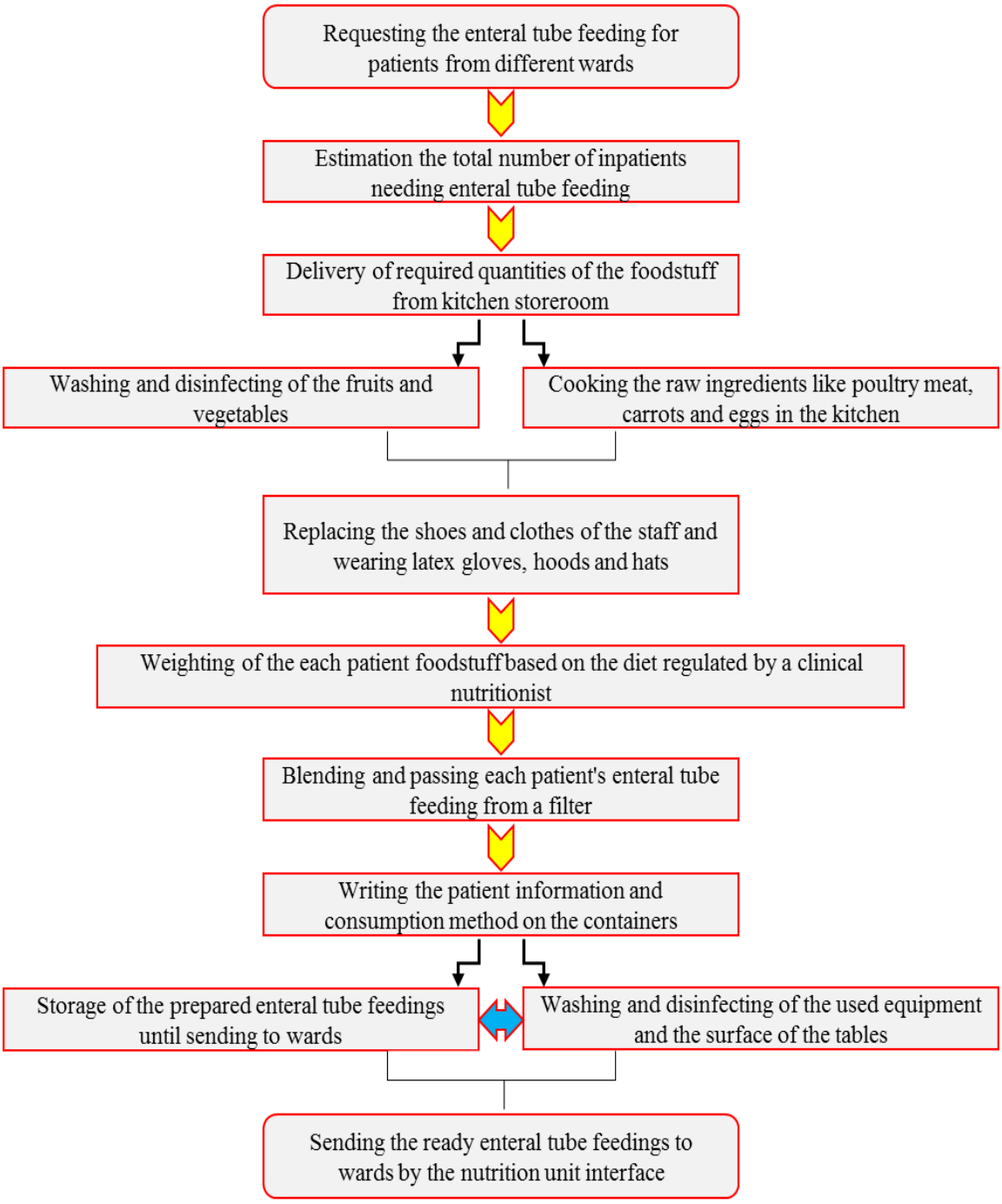

Figure 1. Flowchart of enteral tube feeding preparation process.

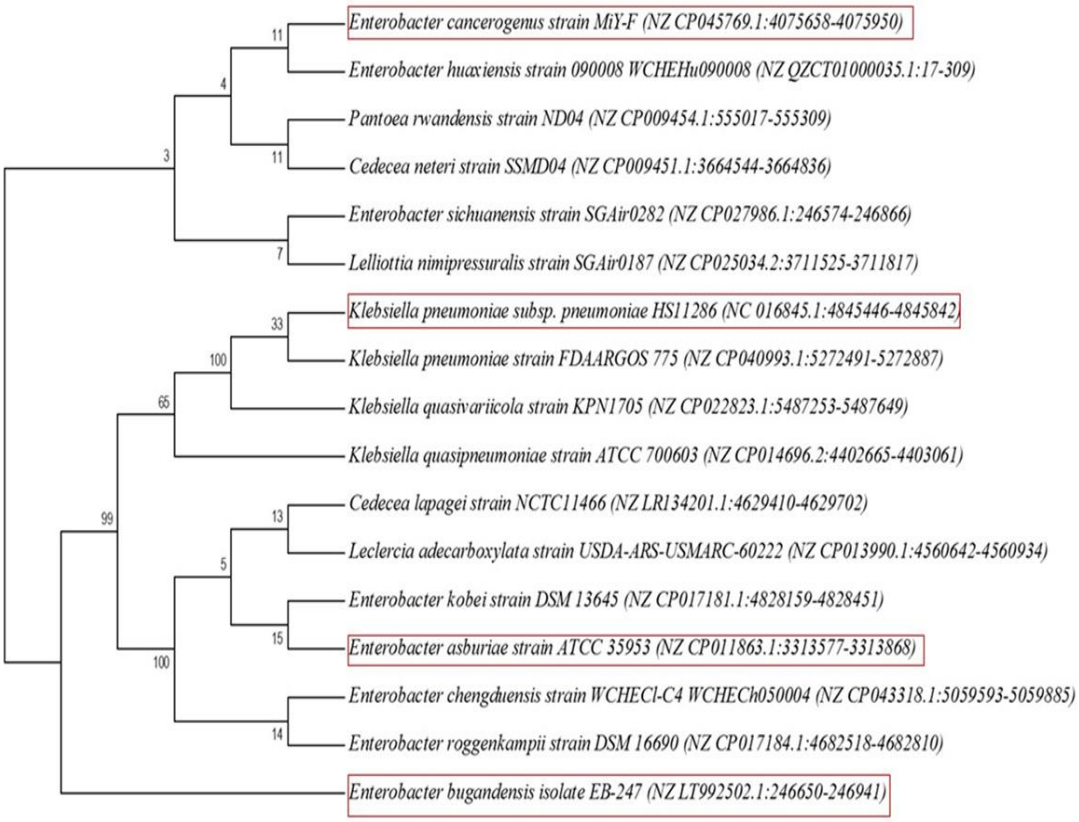

Figure 2. Neighbor-Joining phylogenetic tree resulting from 16S rRNA gene sequence data showing the phylogenetic relationships between isolated *Enterobacter* spp. strains and *Klebsiella pneumoniae*.

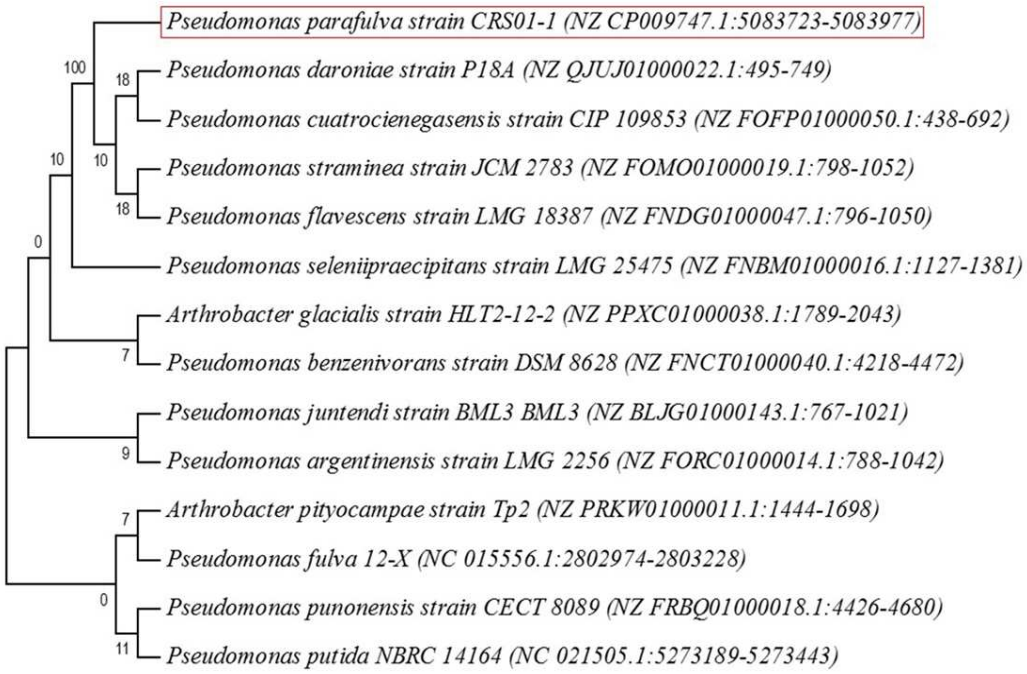

Figure 3. Neighbor-Joining phylogenetic tree resulting from 16S rRNA gene sequence data showing the phylogenetic relationships of *Pseudomonas Parafulva* and its related taxa.

Table 1. Foodstuff used for enteral tube feeding preparation.

Groups	Food material
Meat and proteins	Boiled chicken, egg, beans like lentil, potato
Dairy and milk	Low-fat yogurt, milk powder
Fruits and vegetables	Baked carrot and tomato, raw cucumber, banana, orange or tangerine, apple, kiwi
Fat	Sunflower oil and olive oil
Bread and cereal substitutes	Rice flour, whole grain biscuit

Table 2. The bacterial count of enteral tube feeding samples.

Bacteria	(CFU *⁄mL)	FDA standard (CFU/mL)
Total Mesophilic Bacteria	57 % , $\geq 10^4$	$10^4 >$
Enterobacter asburiae	80 %, $\geq 10^2$	No Standard
Enterobacter bugandensis	77 % , $\geq 10^2$	No Standard
Enterobacter cancerogenus	42 % , $\geq 10^1$	No Standard
Klebsiella pneumonia	37 % , $\geq 10^1$	No Standard
Pseudomonas parafulva	93 % , $\geq 10^3$	No Standard

* CFU; Colony Forming Unit

Table 3. Critical points in the formulation process of enteral tube feeding and the required monitoring system.

Process	Critical Points	Monitoring action
Preparation of raw materials for enteral tube feeding formulation	Personnel hand hygiene and unclean clothes	Inspection of cleaning actions and personnel health card check
Blending of ingredients	Contaminated mixers, equipment, and water	Components expiration date control and disinfection of utensils
Pouring the enteral tube feedings with a plastic pitcher into containers	Transferring contamination due to old and plastic pitcher	Regular microbial test of utensils by the environmental health unit
Enteral tube feedings storage in the kitchen until transporting to the wards	Room temperature leading to the growth of spoilage and pathogenic bacteria	Control of refrigerator temperature