Non-fecal and fecal coliform tests of ready-to-eat food and drinks using fluorogenic and chromogenic media

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Abstract. The aim of this research was to assess the microbiological quality of ready-to-eat foods and drinks. Fifteen samples were enriched in buffered peptone water (BPW) before tested for the presence of coliforms using two chromogenic media (Chromocult Coliform Agar-Enhanced Selectivity (CCA-ES) and Harlequin E. coli agar (HEC)) and a fluorogenic medium (Readycult Coliform 100 (RC 100)) at 37°C. Results showed that all samples contained non-Escherichia. coli coliforms and 12 of them contained Escherichia coli. Twelve E. coli strains and 15 non-E. coli coliform isolates were isolated. Fecal coliform tests showed that the isolated E. coli from 12 samples were of fecal origin while only 7 out of 15 coliform isolates were fecal non-E. coli coliforms.

Keywords: coliform test, chromo-fluorogenic media, ready to eat food

1. Introduction
Lack of hygiene and sanitation in foods and drinks may increase the risk of contamination of microorganisms and harmful chemicals. Contamination can occur at all stages of manufacture and presentation of food and beverages. Unhealthy environments will contain more microorganisms and accelerate the growth of these microorganisms. Contaminated food and drink can potentially cause a disease called Foodborne Disease (FBD). Foodborne Diseases can be caused by pathogenic microorganisms that include viruses, bacteria, and protozoa. Supervision of sanitation and hygiene of food and drink needs to be done to prevent FBD [1].

One way to assess the hygiene of a food and beverage product is to examine the content of its microorganisms. This can be done by examining the presence of indicator microorganisms (bioindicators). An indicator is something that has a strong association to a condition until its existence indicates that a condition is very likely to occur. Bioindicators are an important component of hygiene testing and supervision in foods and beverages and the main requirements for food sellers. Bioindicators reflect the quality of a product and determine whether the product is worth selling and consumed by consumers [2]. One of the oldest and most commonly used bioindicators is coliform. Coliforms can indicate the presence of microorganisms that cause FBD. They can be divided into two groups, non-fecal coliforms and fecal coliforms both of which are able to ferment lactose at different temperatures (35 °C and 44.5 °C respectively) [3].

Hasan [4] conducted coliform test to check the hygiene and sanitation of beverages with chromogenic medium. The coliform test showed that 80 % of the samples contained coliforms. Santoso [5] has also conducted coliform tests with the results 16 of 18 samples (88.9 %) contained coliform and 13 of 18 samples (72.2 %) contained E. coli. These results indicate poor hygiene sanitation. Coliform tests should be performed on more foods and beverages to evaluate the likelihood of FBD to occur and need to be held periodically to ensure the microbiological quality of foods and beverages.
Chromocult Coliform Agar-Enhanced Selectivity (CCA-ES) and Harlequin *E. coli* agar (HECA) are chromogenic media that can be used for coliform testing. Testing of ready-to-eat food and drink samples and the characterization of the CCA-ES and HECA isolates obtained in the positive samples are helpful in knowing the sanitary and hygiene conditions of food and beverages. The objective of the study was to examine and analyze the microbiological quality of prepared food and beverage samples using two chromogenic media (CCA-ES and HECA) along with a fluorogenic medium (Readycult coliform 100). The microbiological and analytical qualities are based on the presence of non-fecal and fecal coliform.

There are two hypotheses that can be taken from the microbiological quality of food and beverages. The first, food and beverages do not meet hygienic/microbiological quality. The second one results of the coliform test with CCA-ES and HECA will strengthen and support the results of coliform assays obtained with Readycult Coliform 100 medium.

2. Materials and methods

The study was conducted within a period of 3 months, from September 2016 to December 2016. Samples were obtained from the canteen of one faculty in a campus. All samples were taken within the faculty environment. The necessary tools are laboratory glass equipment (Duran), Micropropette with scale 100, 1000, and 10,000 microliter (CAPP Denmark) along with appropriate tips, digital scales (Shimadzu Model BL-620S), disposable petri dish (Extragen), incubator (Memmert B30), (Leica ICC 50 HD) microscope, Autoclave (Tommy SX500) located in UV lab and handheld (Evaco Universal Money Tester).

A total of 15 samples were obtained. The sample consists of 10 types of food, 4 types of drinks, and tap water. Food samples consist of potatoes, tempeh, fries, chicken opor, cakes, fried noodles, tuna, salad, and sambal. Drink samples consist of mango, apple, star fruit, and avocado juices. The medium used for enrichment was the sterile Buffered Peptone Water (BPW) from Lab M. Nutrient agar (NA) from Merck was used in the rejuvenation of coliform isolates. The required chromogenic medium was Chromocult Coliform Agar-Enhanced Selectivity (CCA-ES) from Merck and Harlequin *E. coli* Agar (HECA) from Lab M. Readycult Coliform 100 from Merck was a fluorogenic medium. Indole Testing uses Kovac’s Reagents (Indole Test) from Merck. Methyl Red test is done using MRVP broth and methyl red reagent. Other chemicals needed for aseptic work were distilled water, 70 % alcohol, and spirits.

Food samples were crushed and weighed. Samples were inoculated into a BPW medium with a ratio of 1:10. A total of 10 g or 10 mL samples were fed into 90 mL BPW to form a ratio of 1: 10. The samples that had been inoculated at BPW were incubated at 37 °C for 24 h and observed. Positive results will show a murky medium [6]. Samples from BPW were tested for coliform. The qualitative coliform test was performed by inoculating the sample to the fluorescent medium that is RC 100. The prepared sample was inoculated to RC 100 with micropipette. RC 100 which was given 1 mL BPW with sample then incubated at 36 °C for 24 hours. The results were then recorded, documented, and visualized in the form of diagrams and photographs. The positive result of RC 100 was the discoloration of the medium into a bluish green that can glow in UV light at a length of 366 nm. *E. coli* positive samples were demonstrated by dripping Kovac’s reagent (2 drops) for Indole test [7]. The qualitative coliform test was also performed by inoculating the sample to the chromogenic medium of CCA-ES and HECA. At CCA-ES and HECA, the prepared sample was inoculated into both media and dispersed by the quadrant streak method. The medium was incubated at 37 °C. The results were then recorded, documented, and visualized in the form of graphs and photographs. The positive results of *E. coli* in CCA-ES medium will show blue / violet colonies whereas in HECA medium will show a greenish-green colony [7,8].

Colony isolation was performed on culture using quadrant streak technique, which showed positive reaction when dispersed to CCA-ES or HECA. The single colony formed from the quadrant streak was inoculated to a tube containing 5 mL NA slant. The isolates were incubated at the same temperature as the coliform test for 24 hours. Rejuvenation of isolates was performed to increase the number of isolate cells for Indole and Methyl Red tests. The rejuvenated isolates were inoculated into MRVP broth and RC 100 5 mL with 1 loop of the isolate. Readycult Coliform 100 was incubated for 24 hours at 44.5 °C. After incubation, the color of Readycult was observed and placed in UV light and given Kovac’s reagent for Indole test. A positive result will show a red ring on the surface of the medium. The isolates for the Methyl Red test were inoculated to MRVP medium and incubated at 35 °C for 2 days. MR test was done by giving Methyl Red reagent as much as 2 drops. Positive results were characterized by a red ring formed on the surface of the medium [7,9,10].

from the sample is fecal fluorescence when exposed to UV light, and a positive E. coli the color of RC 100 medium. 6 of 7 non-coliform isolates were found to be fecal and thermo tolerant isolates. The results of the fecal isolates (100%) can grow at a temperature of 44.5°C. E. coli isolates also showed color change, fluorescence when exposed to UV light, and a positive indole reaction. It shows that all E. coli isolated from the sample is fecal E. coli [7, 9, 10].

Table 1. Samples used for coliform test and the results of enrichment with BPW

| No. | Sample types | Sample items | Bacterial growth in BPW* |
|-----|--------------|--------------|-------------------------|
| 1   | Beverages    | mango juice  | +                       |
| 2   | Beverages    | apple juice  | +                       |
| 3   | Beverages    | starfruit juice | +                   |
| 4   | Beverages    | avocado juice | +                    |
| 5   | Beverages    | tap water    | +                       |
| 6   | Beverages    | fried potatoes | +                    |
| 7   | Beverages    | tempeh       | +                       |
| 8   | Beverages    | spicy potatoes | +                    |
| 9   | Beverages    | chicken opor | +                       |
| 10  | Beverages    | perkedel     | +                       |
| 11  | Beverages    | fried noodle | +                       |
| 12  | Beverages    | spicy tuna   | +                       |
| 13  | Beverages    | salad        | +                       |
| 14  | Beverages    | mayonnaise   | +                       |
| 15  | Beverages    | chili sauce  | +                       |

*Note: + = growth, - = no growth

3. Results and discussions

Samples comprised of ten ready-to-eat foods, four beverages, and tap water. All samples taken can be seen in column 3 of table 1. Tablewares were washed in the same area using the available tap water. The results of enrichment on the BPW medium can be seen in column 4 of table 1. The growth of microorganisms can be seen from the murky medium. Results from observations show that all BPW becomes cloudy after incubation. Samples in BPW showing positive results were further tested to determine the presence of coliform.

The results of the coliform test with RC 100 showed that all of the inoculated media of the sample undergo a medium-color change from yellow and clear to a bluish green. It shows that all samples tested contain coliform. The existence of E. coli is known by exposing RC 100 medium at Ultra Violet light. The results of UV exposure showed that 12 (80%) of the 15 samples fluoresce, thus containing E. coli. The coliform test medium was given a few drops of Kovac’s reagent to see whether or not a red ring appeared on the surface of the medium. Indole results support the results of the coliform test of food and beverage samples because all of the fluorescent samples in UV rays produce Indole rings (containing E. coli), while non-fluorescent samples do not show Indole rings (not containing E. coli). The results of coliform, fluorescent, and indole tests can be seen in table 2.

The sample coliform test was also performed with Chromocult Coliform Agar - Enhanced Selectivity (CCA - ES) medium and Harlequin E. coli agar (HECA). E. coli can be grown differentiated, and separated so that it can be purified. CCA-ES and HECA are differential selective media that can grow colonies of E. coli and non-E. coli coliforms. All samples tested in CCA-ES medium produced red colonies indicating coliform and 80% of the samples yielded red and purple or blue colonies indicating coliform and E. coli. It also resembles a quadrant streak at HECA where all the samples produce salmon-red colonies and 80% of the samples produce salmon-red colonies along with bluish green (E. coli). Both CCA-ES and HECA show that only samples 4, 6, and 14 do not produce E. coli colonies. These results are identical to the results obtained in the coliform test with RC 100 medium. Quadrant streak was performed several times to obtain a pure colony of E. coli. The fecal coliform test with RC 100 was performed to reveal whether the isolates of E. coli and coliform were found to be fecal and thermo tolerant isolates. The results of the fecal coliform test show that 7 of 15 non-E. coli coliform isolates are fecal (46.7%). This was observed from isolates that cannot change the color of RC 100 medium. 6 of 7 fecal coliform isolates show negative results from indole test. All E. coli isolates (100%) can grow at a temperature of 44.5°C. E. coli isolates also showed color change, fluorescence when exposed to UV light, and a positive indole reaction. It shows that all E. coli isolated from the sample is fecal E. coli [7, 9, 10].
Table 2. Results of coliform test using RC 100

| No. | Sample Items       | Readycult Coliform 100* | Fluorescence | Indole |
|-----|-------------------|-------------------------|--------------|--------|
| 1   | mango juice       | +                       | +            | +      |
| 2   | apple juice       | +                       | +            | +      |
| 3   | starfruit juice   | +                       | +            | +      |
| 4   | tap water         | +                       | -            | -      |
| 5   | avocado juice     | +                       | +            | +      |
| 6   | fried potatoes    | +                       | -            | -      |
| 7   | tempeh            | +                       | +            | +      |
| 8   | spicy potatoes    | +                       | +            | +      |
| 9   | chicken opor      | +                       | +            | +      |
| 10  | perkedel          | +                       | +            | +      |
| 11  | fried noodle      | +                       | +            | +      |
| 12  | spicy tuna        | +                       | +            | +      |
| 13  | salads            | +                       | +            | +      |
| 14  | mayonnaise        | +                       | -            | -      |
| 15  | chili sauce       | +                       | +            | +      |

*+) + = positive, - = negative, bold = does not contain E. coli

E. coli produces a positive reaction to the MR test [11]. In the study, the obtained E. coli isolates showed a positive reaction to the MR test. A weak positive reaction (orange) is indicated by isolates from samples 10 and 15. Incubation continues until the fifth day if weaker positive results are observed [10]. The MR test on the isolate was performed again on E. coli isolates from sample number 15 and 10 after incubation for 5 days. MR test showed that E. coli isolates from sample number 15 were acidic and red in color. The E. coli isolate from sample number 10 showed an orange color that turned red when given more MR reagents (5 to 7 drops). These results confirm the coliform test that both isolates are E. coli.

Buffered Peptone Water (BPW) serves to recover cells from stress or pressure caused by the food’s manufacturing/ preparing processes. BPW contains NaCl, which keeps the ionic stability of the medium. Phosphate compounds in the medium act as buffers that keep the pH of the medium neutral (pH 7), while peptone serves as a source of carbon, nitrogen, vitamins, and minerals that can be used by bacterial cells [12]. BPW was incubated at 37 °C for 24 hours. The color change of RC 100 medium is caused by the enzymatic reaction of the 5-bromo-4-chloro-3-indoleyl-ß-D-galactopyranoside (XGAL) substrate in RC 100 medium with the β-galactosidase enzyme owned by coliform. The β-galactosidase enzyme hydrolyzes XGAL substrates that produce chromogenic compounds (indigo blue). The compound changes the color from medium to turquoise and signifies coliform [7]. The content of 4-methylumbelliferyl-ß-D-glucuronide (MUG) substrate on RC 100 medium can only be broken down by E. coli that have the β-glucuronidase enzyme. Methylumbelliferyl-ß-D-glucuronide is broken down into 4 Methylumbelliferone (MU). Methylumbelliferone is a fluorogenic compound that shines under UV light. The sample contains E. coli if the RC 100 medium is fluorescent under UV light [7]. The MUG compound of the MUG solution is a blue fluorogenic compound that can be seen under UV rays (365-366 nm). RC 100 also contains isopropyl-ß-D-galactoside (IPTG), an additional compound that induces the β-galactosidase enzyme to aid total coliform detection [13]. The coliform test medium was given a few drops of Kovac's reagent to see whether or not a red ring appeared on the surface of the medium. Indole red rings are formed by the enzyme tryptophanase produced by E. coli. The content of tryptophan in RC 100 medium was broken down by tryptophanase, thereby forming an indole compound.

CCA-ES contains 2 substrates, namely Salmon Red ß-D-Gal and X-ß-D glucuronide which aims to distinguish E. coli colonies from coliform colonies and other Gram negative bacterial colonies. Coliform bacteria have the enzyme ß-D-galactosidase which can only break down substrate Red Salmon ß-D-Gal. E. coli has ß-D-galactosidase and ß-D-glucuronidase enzymes that break down both substrates. E. coli colonies will form dark blue or violet colonies, other coliforms will form red colonies [7]. In HECA, the ß-Gal Magenta substrate is broken down by bacteria having the ß-
Galactosidase enzyme and the X-glucuronide substrate broken down by the β-glucuronidase enzyme. *E. coli* has both enzymes and forms a greenish-blue colony. Other bacterial colonies that have only β enzymes - Galactosidase will form pink colonies (usually other coliform species), whereas bacterial colonies that do not have both will form white colonies (*Pseudomonas aeruginosa*) [8].

The non-*E. coli* coliform isolate derived from sample number 8 showed an indole ring after a fecal coliform test. The positive indole yields of fecal coliform isolates may be due to uncultivated coliform isolates isolated from *E. coli* bacteria. The fecal coliform isolates may be mixed with *E. coli* thermotolerant bacteria that cannot break down the β-glucuronide substrate and do not fluoresce in UV light nor have colony colors that are different from other coliforms. According to McDaniels *et al.* [14] not all *E. coli* strains found in the environment can express MUG enzymes that break down glucuronide and cause fluorescence under UV light [14]. Other causes of positive indole outcomes are thought to originate from other fecal coliform bacteria, such as *Klebsiella oxytoca*. *K. oxytoca* is a thermotolerant coliform that can produce Indole red rings [9,15].

The positive result of MR test is the presence of red color indicating low pH medium because bacteria have fermented the mixed acid. Some of the genera of bacteria that can perform such fermentation are *Escherichia*, *Salmonella*, *Proteus*, and *Aeromonas* [16]. The MR test results on coliform isolates showed that most of the isolates were negative (did not form red). The coliform isolates of samples 2, 8, 9, and 13 showed positive results. It is thought to occur because one member of the coliforms, *Citrobacter* can lower the pH of the medium, making it acidic. The coliform isolate that showed positive results may contain *Citrobacter* [11]. Based on the coliform and fecal coliform tests that have been done, it has been determined that ready meals, tap water, and beverages do not meet the hygiene standards set by Ministry of Health Regulation No. 1098 which states that the number of *E. coli* bacterial cells in food and drink should be zero (CFU = 0) [17] while total coliform count in beverages should also be zero (CFU = 0) [18]. The presence of non-*E. coli* coliforms in all samples tested showed a low level of sanitation. Through observations that have been conducted, it is known that all the tableware washed in one place using tap water. The results of the coliform test of tap water revealed that the water contained non-*E. coli* fecal coliforms.

The presence of *E. coli* in 80% of samples tested indicates poor hygiene. These results do not meet the standards of the Ministry of Health [19]. *E. coli* found in the sample can also live at elevated temperatures (44.5 °C) indicating that they are fecal (thermo tolerant) and derived from the digestive tract [9]. Contamination of fecal *E. coli* can be caused by foodstuffs and sellers who are not clean or exposed to fecal matter. Using uncooked water in the process of making food and drink also causes contamination. *Escherichia coli* is a bacteria that optimally grows at 37 °C, but can survive at higher (50 °C) and lower (4°C) temperatures [20]. Storing food at room temperature with an open container increases the likelihood of contamination and the growth of *E. coli* and other coliforms in food and beverages. According to the Decree by the Ministry of Health No. 715, perishable foods need to be stored at high (65.5 °C or above) or low temperatures (4°C or lower) [21]. Article 9 of Ministry of Health Decree No. 942 states that food snacks need to be presented in a closed and cleanly wrapped [19].

4. Conclusions
Through the fecal coliform/coliform test, it is known that the microbiological quality of all samples of ready-to-eat foods and beverages did not meet the health standards set by the Minister of Health of the Republic of Indonesia whilst the results of the coliform test with CCA-ES and HEC medium strengthened and supported the test results from the RC 100 medium. Routine coliform/coliform examination must be regularly conducted on more samples in order to assess the microbiological quality of all food and drinks. Coliform and fecal coliform assays should be performed using quantitative methods that can count bacterial colonies in the sample in order to better determine the level of contamination. These are to prevent the possibility of food borne disease due to unhygienic foods and drinks.

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