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USE OF PRINCIPAL COMPONENT ANALYSIS AND AGGLOMERATIVE HIERARCHY CLUSTERING TO STUDY THE RELATIONSHIP BETWEEN ALPHA AMYLASE AND STARCH DURING FERMENTATION OF *OGI* FROM MAIZE, SORGHUM AND *ACHA*

Adekunbi A. Malomo[1], Omowumi I. Olaniyi[1], Abiola F. Olaniran[2], Sunbo H. Abiose[1]*

Address (es):
[1]Department of Food Science and Technology, Obafemi Awolowo University, Ile-Ife, Nigeria., [2]LandmarkUniversity, Omu aran,Kwara State, Nigeria.

*Corresponding author: oibipejuolaniyi@gmail.com

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ABSTRACT

Ogi, a popular breakfast cereal and weaning food in West Africa was produced from maize, acha and sorghum. *Ogi* was produced from the three cereals and the gruels obtained were subjected to fermentation. The total starch content and alpha amylase activity during the period of fermentation of the *ogi* slurries were evaluated using standard methods. Total starch content was determined at 0, 12, 24, 36 and 48 h and α-amylase activity was also determined at 10 ºC, 20 ºC, 30 ºC, 40 ºC at 0, 12, 24, 36 and 48 h. The outcome of the research showed that α-amylase activity increased with increase in temperature and was highest in maize *ogi* (1.507 – 4.458 E. U) while acha *ogi* (0.497 – 3.219 E. U) had the lowest α-amylase activity . The starch content decreased with increase in time of fermentation time and was highest in maize *ogi* while acha *ogi* had the lowest. This shows that the higher the temperature of fermentation and longer fermentation time can increase the breakdown of starch by α-amylase in *ogi* slurry. The result of the Principal component analysis (PCA) showed distinct similarity in maize and acha. Agglomerative hierarchical clustering grouped acha into two groups (groups one and two) but grouped all maize and sorghum *ogi* samples together into group three. Due to the low starch content and low α-amylase activity observed in acha, it could be utilized in the production of gluten-free *ogi* with low glycemic index especially for vulnerable groups.

Keywords: alpha-amylase, starch, fermentation, maize, sorghum, acha, *ogi*, PCA, hierarchy, clustering, relationship

INTRODUCTION

Ogi is a smooth, free flowing thin porridge obtained from wet milled, fermented maize and sorghum in the southern part of Nigeria. It serves as a major weaning food for infants, a breakfast meal for both children and adults in West Africa **(Abioye and Aka, 2015; Ukeyima *et al*., 2019)**. *Ogi* can be produced from cereals such as maize, sorghum, millet and recently acha. The choice of grain depends on preference and ethnicity **(Ohenhen and Ikenebomeh, 2007)**.
Presently, researchers are studying how resistant starches can reduce the glycemic and insulin response **(Deepa *et al*. 2010; Jideani and Jideani, 2011)**. The in-vitro starch digestibility and glycemic property of acha and maize porridge has been investigated.The in-vitro digestibility and glycemic property of maize and acha showed the digestible starches (DS) in maize and acha were 43.7 and 41.4 **(Jideani and Podgorski 2009)**. Acha also contains resistant starches which has been shown to be promising in the management or prevention of certain diseases or health conditions **(Deepa *et al*. 2010; Jideani and jideani, 2011)**.
Fermentation improves absorption of nutrient, especially from agricultural produce, by enzymatic splitting of cellulose, hemicellulose, and related polymers that are not digestible by humans into simpler sugars and sugar derivatives **(Parker, 2018; Malomo *et al*., 2019)**. Most of these enzymes are naturally present in cereal grains but at low levels **(Malomo *et al*., 2019)**. α-Amylases are hydrolytic enzymes which specifically break the α-1, 4-glucosidic bonds in starch. Possible sources of α-amylase in plants, animals and microbes have been reported **(Aiyer, 2005; Asante *et al*., 2013)**. The effect of controlled fermentation of *ogi* has been reported **(Ohenhen and Ikenebomeh, 2007)**. The volatile compounds and associated microbes during fermentation of *ogi* slurry from maize have been identified by **Bolaji *et al*. (2020)**. There are several articles on the use of acha but there is a dearth of information on the effect of fermentation on α-amylase activity and starch during fermentation of *ogi* from acha, sorghum and maize, hence this study.

MATERIALS AND METHODS

White Quality protein maize variety (ART/98/SW06/OB/W) was obtained from the Institute of Agricultural Research and Training (I.A.R.T.), Ibadan, Nigeria. Sorghum (red variety) was purchased from a local market in Ile-Ife, Osun State, Nigeria. Acha grains were purchased from a local market in Ebonyi State, Nigeria.
Ogi was produced from the cereals using the method described by **Olaniran *et al*. (2019)**. Slurries of *ogi* obtained from these cereals were further fermented for 48 h. Samples were taken from the fermenting slurries at 0, 12, 24, 36 and 48 h. Samples obtained were then used for further studies.

Determination of α-amylase activity of *ogi* samples

Alpha-amylase activity of the fermenting *ogi* samples were determined as described by **Malomo *et al*. (2019)**. Each *ogi* sample (5 g) was weighed at 0, 12, 18, 24, 36 and 48 h of fermentation and homogenised in 50 ml of 0.2 M sodium acetate buffer (pH 4.0). The homogenate was transferred into a conical flask and was mechanically shaken at 150 rpm for 10 min at room temperature in a Gallenkamp orbit shaker (3597 C2-2, England). The suspensions were transferred to centrifuge tubes and centrifuged at 5000 rpm for 30 min in centrifuge (Bosch Model No TDL-5, Germany) and the supernatant was used as crude enzyme for enzyme assay. The substrate for assay was 0.5 ml of 0.5 % soluble starch, buffered with 0.2 ml of 0.2 M sodium acetate (pH 5.6). Crude enzyme extract (0.3 ml) was added to the mixture, mixed and incubated at 40 °C for 30 min in Gallenkamp water bath (HH-S6, England). The reaction was terminated by the addition of 2 ml of 3, 5-Dinitrosalicyclic acid (DNSA) and boiled for 5 min in the water bath. The mixture was cooled under running water and 7 ml of distilled water was added. Blank that consisted of 0.3 ml distilled water, 0.5 ml of 0.5 % soluble starch and 0.2 ml of buffer was treated in similar way. The absorbance of the resultant solution was read at 540 nm in UV Spectrophotometer (Spectrumlab 752S, YM1206PHB2, China). Reducing sugar in the samples was estimated from a standard curve of known concentrations of maltose (0-1000 μg/ml). One unit of α-amylase was defined as the amount of enzyme required to produce 1 microgram of reducing sugar equivalents per minute measured as maltose from soluble starch under the experimental conditions. 1 enzyme unit (E.U.) =1 μg of maltose produced/min

Extraction and estimation of starch from *ogi* samples

Modified method of **Kiran and Sandeep (2016)** was used for determination of starch by using anthrone method. *Ogi* samples (5 g) was homogenized in 80% ethanol to remove sugars present in the samples. Residue was retained after centrifugation at 5000 X g for 15 min. The starch was extracted by 52% perchloric acid at 0 ºC for 20 min. One millilitre (1 ml) of extract fromeach sample was taken into testtubes and 4 ml of anthrone was added. The tubes were kept for boiling for 5 min. The colour intensity was measured at 620 nm in spectrophotometer (Spectrumlab 752S, YM1206PHB2, China). Starch concentration was calculated from standard curve of known concentration glucose (0 – 10000μg).

RESULTS AND DISCUSSION

Alpha amylase activity during fermentation of *ogi* samples

The α-amylase activity increased with increase in temperature (Fig. 1-4). It was generally higher in *ogi* samples produced from maize (1.507- 4.458 E. U) than

sorghum (1.070 – 3.019 E. U) and lowest in *ogi* produced from acha (0.497 – 3.219E. U) throughout the period of fermentation.It was within the range of 0.497 – 3.281 E. U at 10 ºC, 0.643 – 3.453 E. U at 20 ºC, 0.931 – 3.531 at 30 ºC and 1.875 – 4.458 E. U at 40 ºC. Acha was reported to have lower α-amylase activity during fermentation of *the ogi* slurries and relatively evoke low sugar on consumption, an advantage for diabetics **(Malomo *et al.,* 2019)**. Amylolysis susceptibility of maize starch is higher, because of the presence of surface pores and channels that facilitate enzymatic diffusion **(Zhang *et al*. 2006)**.

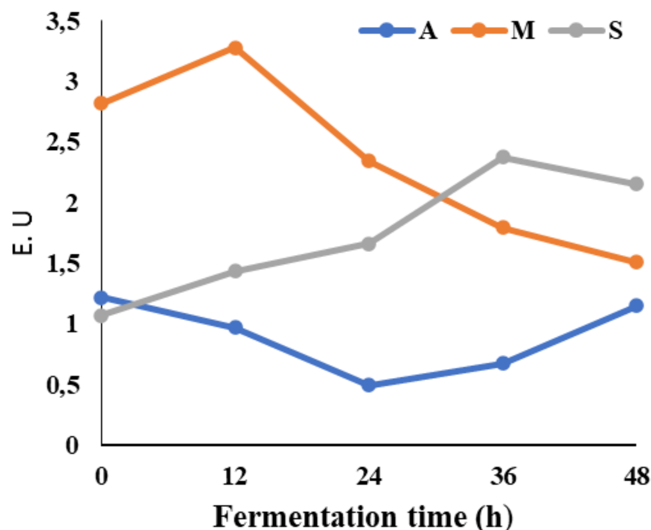

Figure 1 Alpha amylase activity of *ogi* samples at 10 ºC

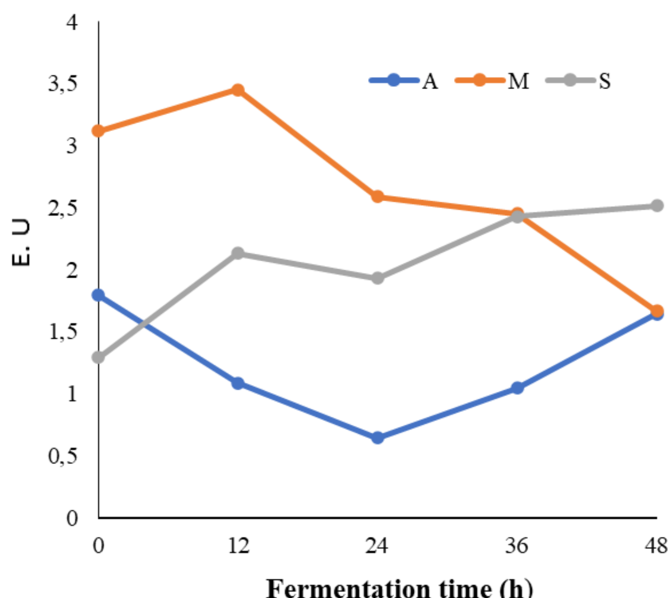

Figure 2 Alpha amylase activity of *ogi* samples at 20 ºC

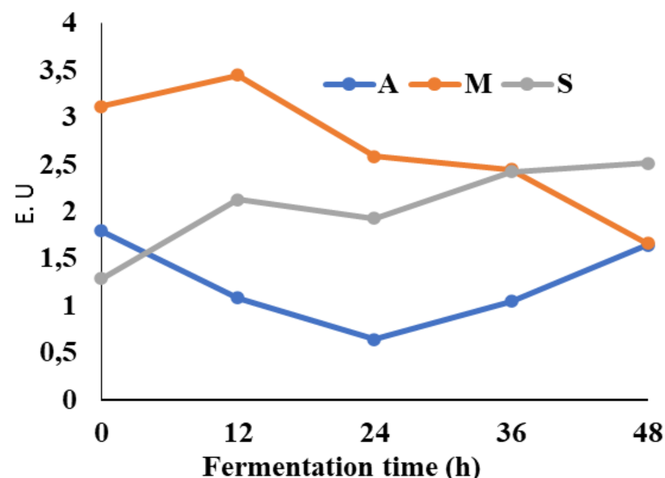

Figure 3 Alpha amylase activity of *ogi* samples at 30 ºC

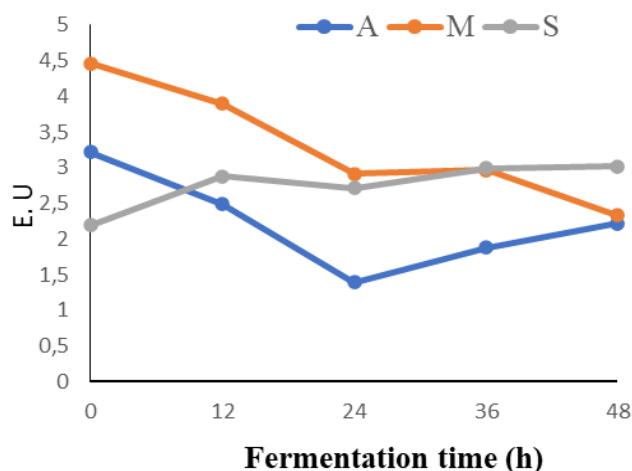

Figure 4 Alpha amylase activity of *ogi* samples at 40 ºC

Total starch content of *ogi* samples

Ogi samples produced from maize had the highest starch content while (61.20 – 71.55 %) and lowest in acha *ogi* samples (33.31 – 51.86 %) (Table 1).There was significant difference ($p < 0.05$) in the starch content of all the cereals from 0 – 36 h of fermentation but there was no significant difference ($p > 0.05$) between *ogi* from maize and sorghum *ogi* at 48 h of fermentation. Starch is the main constituent of maize kernels, about 72–73% of the total weight (Paraginskia *et al*. 2014). Acha (68 %) is reported to have lower starch content than sorghum (73.8 %) **(Ballogou *et al*., 2013)**.

Table 1 Changes in total starch content of *ogi* samples

Sample	Fermentation time (h)				
	0	12	24	36	48
A	51.86^{c}±1.200	51.32^{b}±1.11	47.06^{c}±2.01	42.47^{c}±1.78	33.31^{b}±0.70
M	71.55^{b}±1.00	69.16^{b}±0.08	68.45^{b}±0.65	66.58^{b}±1.03	61.20^{a}± 1.50
S	73.23^{a}±0.56	72.96^{a}±0.74	72.11^{a}±1.02	70.99^{a}±0.98	60.00^{a}±0.02

Keys: A- *Ogi* from Acha; M- *Ogi* from Maize; S- *Ogi* from Sorghum

The principal component analysis (PCA) was used to determine the relationship between temperature and α-amylase. Also, to evaluate the effect of α-amylase on starch during fermentation of *ogi* produced from maize, acha and sorghum. The principal components were grouped into five components and PC 1 and PC 2 best represent the samples.

Starch (TS) had positive correlation (Fig. 4) with all the variables but had strongest correlation with α-amylase activity at 10 ºC (0.616) and reduced to 0.544 at 40 ºC. This showed that as the temperature increased, the starch production decreased. The temperature at 10 ºC had strongest positive correlation at 20 ºC (0.969) and also reduced progressively to 0.658 at 40 ºC.

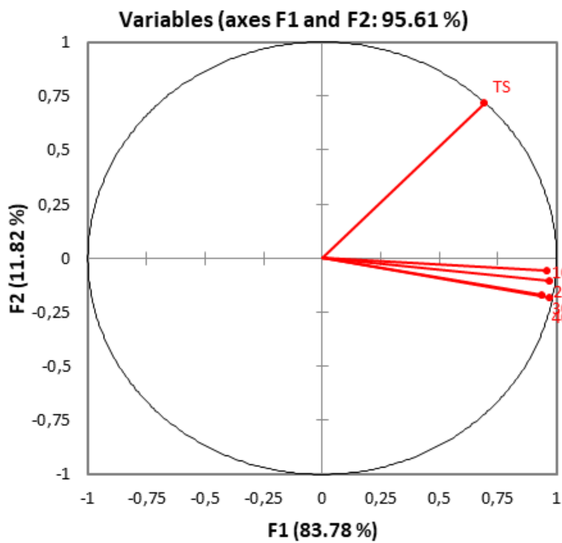

Figure 5 Principal component analysis (PCA) for parameters-projection of variables in the factor-plane, considering two factors.

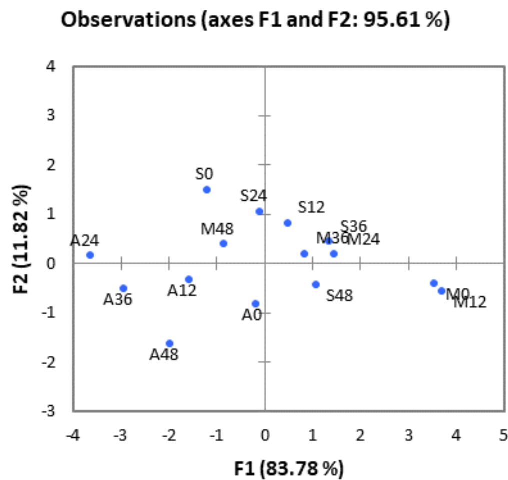

Figure 6 Principal component analysis (PCA) for parameters-projection of observation in the factor-plane, considering two factors.

The relationship between the samples are shown in Fig. 6. All acha samples were grouped separately on the negative side of PCA 1 except A0 at the negative side of PCA 2. Maize *ogi* fermented for 0 to 36 h were represented on the positive axis of PCA 1 and M48 on the positive side of PCA 2. Sorghum was represented on the positive axis of PCA 2 at 0 – 24 h and on the positive side of PCA 1 from 36 to 48 h. This showed that there is similarity in the pattern of α-amylase activity in both maize and sorghum *ogi* during fermentation.

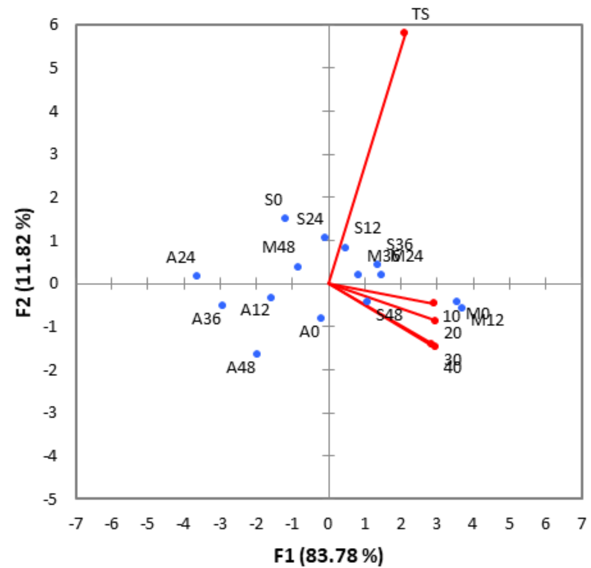

Figure 7 Principal component analysis (PCA) for biplot in the factor-plane, considering two factors.

The biplot (Fig 7) showed that the α-amylase activity on maize *ogi* and sorghum *ogi* fermented from 0 to 24 h had positive correlation with TS while all *ogi* samples produced from acha had negative correlation with TS which means that *ogi* from acha had the lowest TS. The lower the temperature, the stronger the correlation with starch. This showed that increase in α-amylase activity led to decrease in starch.

Agglomerative hierarchical clustering (AHC) (Fig. 8) S grouped *ogi* samples into three. Acha *ogi* fermented from 0 to 36 h were grouped in class 1 while at 48 h, acha *ogi* was grouped in class 2. Maize *ogi* and sorghum *ogi* were grouped in class 3 showing that there are similarities between alpha amylase activity and starch content of maize *ogi* and sorghum *ogi*.

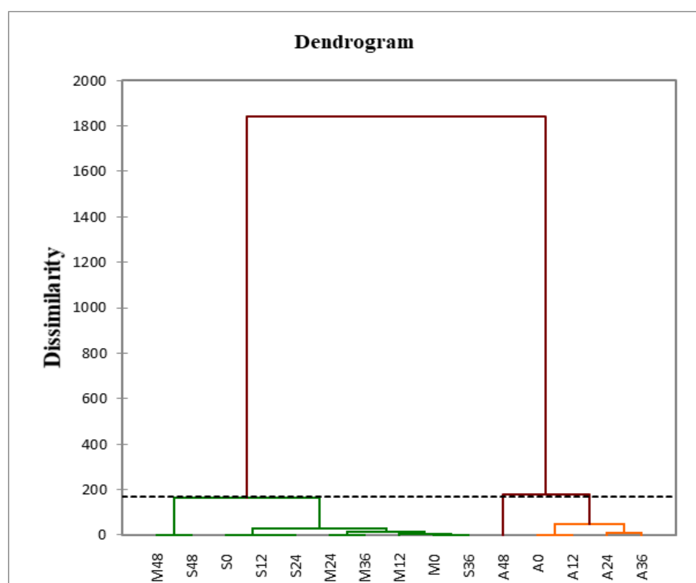

Figure 8 Agglomerative hierarchical clustering

CONCLUSION

The α-amylase activity generally decreased with increase in temperature. It was highest in maize *ogi* and lowest in acha *ogi*. Total starch content was also highest in maize *ogi* and lowest in acha *ogi*. PCA showed more similarity in maize *ogi* and sorghum *ogi* than acha *ogi*. The AHC grouped *ogi* produced from acha in different and grouped acha *ogi* separately. It is therefore suggested that *ogi* with low glycemic index could be produced from this rich non-glutenous cereal "acha".

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ACTIVITY OF KEFIR GRAINS AGAINST *GARDNERELLA VAGINALIS*

Salwa S. Afifi[1], *Amany A. El-Sharif*[1], *Zeinab H. Helal*[1*], *Eman R. El-Musallamy*[1, 2]

Address (es):
[1]Department of Microbiology and Immunology Faculty of Pharmacy, Al-azhar University, Cairo, Egypt.
[2]Gynecology Clinic, El-Monib Family Medicine Center, Giza, Egypt.

*Corresponding author: zeinabhelal@hotmail.com

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ABSTRACT

Kefir is a probiotic mixture of bacteria and yeasts. In vitro and animal trials have shown kefir to have antibacterial, antifungal and antiviral activity. The main goal of this study was to determine the antimicrobial susceptibility of *Gardnerella vaginalis* (*G.vaginalis*) to kefir. Antimicrobial agents, which were commonly used to treat bacterial vaginosis, and kefir were both tested for antimicrobial activities against *G.vaginalis* using an agar diffusion method. Kefir was able to inhibit the growth of *G.vaginalis*. Kefir found to have antimicrobial activity similar to ampicillin, chloramphenicol, ceftriaxone, erythromycin and gentamicin against *G.vaginalis* isolates. Kefir produced zone of inhibition sizes higher than the zones produced by ciprofloxacin, sulphamethoxazole /trimethoprim and tetracycline. Special attention must be paid to kefir as its activity against *G. vaginalis* recommends that it deserves assessment in the treatment of infections involving *G. vaginalis*.

Keywords: Kefir, *Gardnerella vaginalis*, Agar diffusion method

INTRODUCTION

Bacterial Vaginosis (BV) is the most common cause of vaginal discharge and characterized by shift of microbiome like Lactobacillus species by opportunistic pathogenic bacteria. BV has been connected to severe gynecological and obstetric problems; it is associated with preterm delivery (**Stevens et al., 2004**). Moreover, BV increases the chance of spread and acquisition of sexually-transmitted diseases, for example HSV-2 (**Cherpes et al., 2005**) and HIV (**Cohen et al., 2012**). Generally, *Gardnerella vaginalis* (*G. vaginalis*) was believed to be the solitary causal agent of this disorder (Catlin, 1992), however its role in the etiology of BV was reduced over the years as the excess of other bacterial species was increasingly associated with the illness (**Machado et al., 2013; Alves et al., 2014; Castro et al., 2019**). Many studies have been placed *G. vaginalis* in the highlight. Several researchers reported that *G. vaginalis* had a significantly higher virulence potential than many other BV-associated bacteria, because of its greater tendency to form a biofilm, complex early adhesion and produce cytotoxic effect (**Machado et al., 2013; Alves et al., 2014**). These findings suggest that *G. vaginalis* may have a principal role in the BV infection development, flagging the way for several opportunists to colonize the vagina (**Machado et al., 2013; Alves et al., 2014; Castro et al., 2019).**

Owing to the complex polymicrobial nature of BV, traditional treatment with antibiotics are notorious for their low efficacy and high rates of recurrent infection (**Bradshaw et al., 2006**; **Bostwick et al., 2016**).

Inadequate functioning of antibiotics is believed to be due to their failure to complete eradication of BV-associated pathogens; because of emerging antibiotic resistance and to their adverse effect on healthy vaginal microbiota (**Bradshaw et al., 2006; Bostwick et al., 2016).** For these reasons, alternative therapeutic agents need to be pursued for the treatment of BV.

Probiotic strains are claimed to enhance health through immunomodulatory, metabolic and improve epithelial barrier activities against pathological processes. Probiotics are live microorganisms which, when administered in adequate amounts, confer a health benefit on the host **(FAO/WHO, 2001**). Many probiotic bacteria produce a broad range of effective antimicrobials, including bacteriocins, lactic acid, and hydrogen peroxide.

Kefir is an example of a probiotic mixture of bacteria and yeasts. Kefir strains have revealed various antifungal and antibacterial activities (**Bourrie et al., 2016**). The antimicrobial effect of Kefir fermented milk has been tested against a wide range of pathogenic bacterial and fungal species (**Bourrie et al., 2016**) and found to have antimicrobial activity identical to ampicillin, amoxicillin, azithromycin, ceftriaxone, and ketoconazole against many of these species (**Bourrie et al., 2016; Rodrigues et al., 2005**).

To our knowledge, there are no reports to date that have investigated the antimicrobial effect of kefir against *G. vaginalis.* This study was designed to isolate the causative agent *G. vaginalis* from bacterial vaginosis patients with their antibiotic sensitivity pattern and to determine the antimicrobial activity of kefir against *G. vaginalis* and compare them with the activity of antibiotics.

MATERIALS AND METHODS

Clinical setting and study population

Specimens were collected from 360 pregnant and non-pregnant women within the age of 18-45 years. Patients, who were diagnosed with malignancy, AIDS, menstruation, were not included in the study. In addition, patients having history of vaginal douche on the day of sample collection or those who had antimicrobial agents within the last thirty days were not included. This study was conducted in patients who attended El-Monib Family Medicine Center, El-Giza, Egypt, Gynecology Clinic; the specimens were submitted to the Microbiology Laboratory, El-Monib Family Medicine Center, El-Giza, Egypt, for routine culture.

Diagnosis for BV and isolation of *G. vaginalis*

Diagnosis of BV was done based on Amsel's clinical criteria; by the presence of at least three of the following criteria: pH > 4.5, positive amine test, homogeneous vaginal discharge, and presence of clue cells. In addition, BV was diagnosed based on Nugent criteria, and later bacteria were cultured on agar. Specimens were cultured on blood agar, sabaroud dextrose agar, and for *G. vaginalis* Columbia blood agar base with *G. vaginalis* selective supplement was used. All the isolates were identified by standard biochemical methods.

Antibiotic susceptibility testing

Since guidelines or consensus were unavailable for antimicrobial susceptibility patterns of *G. vaginalis*, the antimicrobial described for anaerobic bacteria by the CLSI document M100S (**CLSI, 2016**) were used.

All the isolates of *G. vaginalis* obtained by culture were tested for antimicrobial susceptibility by the single disc diffusion method. *G. vaginalis* cells suspension was adjusted to 0.5 McFarland standard solutions. The cultures were tested against ampicillin (AMP; 10µg), ceftriaxone (CRO; 30 µg), chloramphenicol (C; 30 µg), ciprofloxacin (CIP; 5 µg), clindamycin (CD; 2 µg), erythromycin (E; 15 µg), gentamycin (GN; 10 µg), metronidazole (MTZ, 50 µg), sulphamethoxazole/trimethoprim (SXT; 1.25/23.7 µg), and tetracycline (TE; 30 µg).

Antimicrobial susceptibility of Kefir

Kefir starter strains were obtained from Yogourmet Company (Canada, Batch # Q06581). The media for activation of kefir grains was prepared by 100 grams of skim milk to a liter of distilled water, autoclaved at 121°C for 15 minutes, and later cooled to 18-20°C. Twelve gram of Kefir starter per liter was added incubated at 18-20°C for 24 hours.

Evaluation of antimicrobial activity of Kefir was done by using the disc diffusion method. Kefir was pipetted on a 5 mm sterile filter paper disc at the amount of 0.1 ml of 1.2 mg/ml of kefir as described by Rodrigues et al., (**Rodrigues et al., 2005**). Disks were applied to the surface of the Human Blood Bilayer Tween agar media earlier swabbed with organism suspension adjusted to 0.5 McFarland

standard solutions. Inoculated plates were incubated under anaerobic conditions at 37°C for 24 hours. Experiments were performed in triplicates, and mean values were calculated.

RESULTS

A total of 360 women suspected for BV that were between the the ages of 18 to 45 years old were included in this study. Out of 360 patients, based on Amsel criteria, 38 (10.6%) cases were BV positive, and 89.4% were BV negative. Based on Nugent criteria, 39 patients (10.8%) were BV positive, and 89.2% were BV negative. Microbial culture of vaginal fluid yielded growth of *Candida ablicans* in 81 patients (22.5%), followed by *G. vaginalis* (n=39, 10.8%), then *E. coli* (n=21, 5.8%), and *Neisseria gonorrheae* (n=9, 2.5%). Three patients (0.8%) had mixed infection of Candida and *E. coli*. Two hundred and seven (57.5%) cases were culture negative.

The sensitivity pattern of *G. vaginalis* isolates showed that 94.7% of isolates were resistant to tetracycline, 74.4% to sulphamethoxazole/trimethoprim, 64.1%% to ciprofloxacin, and 41.0% to erythromycin. On the other hand, *G. vaginalis* were 100% sensitive to clindamycin followed by metronidazole (89.7%), then gentamycin (82%), ampicillin (76.9%) and chloramphenicol (74.4%), table 1.

Table 1 Antimicrobial susceptibility pattern of *G. vaginalis* isolated in the study

Antimicrobial agents	**Sensitive No. (%)**	**Intermediate No. (%)**	**Resistance No. (%)**
Ampicillin	30 (76.9%)	2 (5.1)	7 (18%)
Cefotriaxone	35 (89.7%)	1 (2.6)	3 (7.7 %)
Chloramphenicol	29 (74.4%)	5 (12.8%)	5 (12.8%)
Ciprofloxacin	14 (35.9%)	0	25 (64.1%)
Clindamycin	39 (100%)	0	0 (0.0%)
Erythromycin	24 (61.5%)	7 (17.9%)	8 (20.5%)
Gentamycin	32 (82.0%)	1(2.6%)	6 (15.4%)
Metronidazole	35 (89.7%)	0	4 (10.3%)
Tetracyclin	2 (5.3%)	0	37 (94.7%)
Sulfamethoxazole/trimethoprim	10 (25.6%)	0	29 (74.4%)

Table 2 The inhibition zones (mm) of kefir and antimicrobial agents against G. *vaginalis* isolates

Isolate #	Kefir	Amp	CRO	C	CIP	CD	E	TE	GN	MTZ	SXT
1	17	19	25	19	23	30	24	0	18	22	12
2	20	20	27	20	13	27	26	0	19	25	10
3	18	19	22	19	12	27	23	0	17	24	9
4	13	13	24	21	14	24	24	0	10	19	8
5	18	19	29	20	22	26	23	0	16	22	11
6	17	18	23	19	10	27	20	0	17	21	8
7	16	19	25	18	25	23	26	0	19	20	20
8	20	20	24	19	9	27	19	0	18	25	8
9	19	21	28	22	8	24	24	0	18	22	7
10	21	21	22	20	22	27	25	0	16	25	23
11	16	17	26	21	22	27	23	0	16	19	10
12	20	22	27	22	13	25	26	0	20	23	19
13	13	15	24	16	22	24	23	0	11	19	21
14	12	13	22	19	9	25	20	0	8	18	18
15	19	21	27	21	7	24	19	0	18	22	0
16	16	17	26	13	0	26	0	0	15	19	0
17	18	21	23	19	10	25	9	0	19	22	9
18	20	20	25	21	27	28	26	0	17	23	18
19	19	22	21	22	22	26	23	19	20	23	20
20	17	18	22	19	7	26	17	0	16	19	8
21	18	18	23	18	12	27	25	0	17	20	9
22	19	20	25	20	11	29	23	0	20	20	7
23	20	21	22	21	13	27	26	0	20	22	19
24	18	19	21	17	21	28	24	0	17	19	18
25	20	20	22	20	12	30	23	0	19	21	9
26	17	19	21	20	22	26	21	0	17	19	11
27	11	12	12	0	8	25	8	0	0	17	7
28	20	20	22	15	0	28	0	0	14	22	0
29	19	21	23	19	24	26	23	20	17	24	19
30	18	19	21	20	21	23	23	0	19	19	10
31	19	19	24	20	12	26	24	0	18	23	8
32	20	22	22	20	24	26	28	0	19	24	10
33	20	21	21	19	22	24	24	0	18	23	11
34	17	16	21	18	12	22	19	0	15	18	8
35	10	9	10	0	0	23	0	0	8	14	0
36	18	19	19	12	0	23	0	0	18	19	0
37	0	0	11	0	0	22	0	0	0	11	0
38	16	18	24	0	0	21	0	0	17	16	0
39	21	22	21	16	10	26	9	0	18	22	9
Mean value	17.2 ±3.9	18.23 ±4.15	22.49 ±4.1	17.1 ±6.2	13.4 ±8.2	25.64 ±2.13	18.5 ±9.1	1 ±4.4	15.87 ±4.77	20.64 ±3	10.1 ±6.7

Legend: Amp: Ampicillin, CRO: Cefotriaxone, C: Chloramphenicol, CIP: Ciprofloxacin, CD: Clindamycin, E: Erythromycin, GN: Gentamycin, MTZ: Metronidazole TE: Tetracyclin, SXT: Sulfamethoxazole/trimethoprim

Table 2. Zones of inhibition diameters of kefir suspension (0.1mL culture grown for 24 h), and the antimicrobial agents tested. Results showed the activity of kefir against 97.4% of the tested isolates. Ampicillin, chloramphenicol, ceftriaxone, erythromycin and gentamicin disks produced zone sizes generally similar to those

of the kefir suspension. Kefir disks produced zone sizes higher than the zones produced by ciprofloxacin, sulphamethoxazole /trimethoprim and tetracycline disks. The mean values of the kefir inhibition zones were 17.2, standard deviations 3.9 mm and medians of 18 mm.

DISCUSSION

Globally, BV causes genital problems among women that require gynecological care. BV has gained clinical importance since the condition has been associated with an increased susceptibility to HIV, herpes simplex viruses, sexually transmitted infections, and human papillomavirus (**Cherpes et al., 2005; Cohen et al., 2012**).

Several potential microbial pathogens have been involved in the BV development. Recent studies have reported that *G. vaginalis* can be a primary pathogen of BV disorder (**Machado et al., 2013; Alves et al., 2014; Castro et al., 2019**). The treatment and control of BV can be employed by reducing the inhabitants of anaerobic bacteria, possibly prompting an increase in H_2O_2-lactobacilli-producing species (**Giraldo et al., 2007**). The antimicrobials recommended for BV are metronidazole and clindamycin (**CDC, 2015**). The efficacy of the afore said treatments are not optimal, as a high degrees of relapse and bacterial antibiotic resistance is commonly reported. The current study evaluated the antimicrobial susceptibility patterns in *G. vaginalis* to antibiotics that are recommended in routine BV treatment empirically and regionally. *G. vaginalis* isolates were found to have resistance to tetracycline (94.7%) and sulphamethoxazole/trimethoprim (74.4%) and were 100% sensitive to clindamycin followed by metronidazole (89.7%). Even with the availability of effective antibioticsfor the treatment of BV, recurrence occurs within a year in approximately 60% of the BV cases (**Bradshaw et al., 2006**). Moreover, there was great concern regarding antimicrobial treatment as it is related to the suppression of vaginal microbiome, which is essential for homeostasis of the vaginal microbiota (**Gajer et al., 2012**). Besides, the recommended therapy, metronidazole and clindamycin, are associated with side effects, including gastrointestinal effects (**Brzezinski et al., 2018**). Consequently, novel therapeutic approaches are required for more success in the treatment of BV. The use of probiotics has received attention as a means of a treatment and preventve option for vaginal disorders. Kefir is the one of the probiotics claimed to have clinical effects on urinary tract infections, diarrheal diseases, *Helicobacter pylori*, streptococcal, and salmonella infections (**Bourrie et al., 2016**). Moreover, kefir grains have been used successfully to treat vaginal infections, due to its ability to produce antimicrobial compounds (**Bourrie et al., 2016, Brzezinski et al., 2018**). Although antimicrobial activity of kefir against many pathogen has been reported (**Bourrie et al., 2016**), antimicrobial activity of the kefir grains against *G. vaginalis* have not been previously described. This work has shown kefir activity against *G. vaginalis* isolates from BV patients. The kefir displayed activity against 97.4% of *G. vaginalis* isolates with zones of inhibition ≥10 mm. Inhibition zones of kefir suspension in this study were of similar size as those reported against *S. aureus*, *E.coli, Salmonella* species, *shigella* species *and bacillus* species (**Garrote et al., 2000; Mohammed & Twaina , 2017**). On the other hand, the study by Guzel-Seydim et al., (**Guzel-Seydim et al., 2016**), evaluated the antimicrobial activity of kefir against *Fusobacterium nucleatum*, they reported that the maximum inhibition zones was 9.5mm.

The plausible effects of the Kefir could be from the production of carbon dioxide, hydrogen peroxyde, bactericides, organic acids and ethanol during fermentation process. Additionally**,** the antimicrobial activity of kefir are owed to lactic acid and antibiotics produced by bacteria and yeasts (**Bourrie et al., 2016**).

CONCLUSION

The data presented in this work suggest that kefir may be good antimicrobial agent for use in *G. vaginalis* infections which is a leading cause of BV. Therefore, kefir could be a promising alternative for protection against *G. vaginalis* in women, thus preventing BV. However, further investigation with in vivo studies is required.

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TEXTURIZED SOY PROTEIN AS AN ALTERNATIVE LOW-COST MEDIA FOR BACTERIA CULTIVATION

Carlos Henrique da Silva Cruz[1], Jessica Bezerra dos Santos[1], Francielle Penha dos Santos[1], Gabriel Macedo Magalhães Silva[1], Eduardo Felipe Nascimento da Cruz[1], Gustavo Luis de Paiva Anciens Ramos[2] & Janaína dos Santos Nascimento[1]

Address (es):
[1] Laboratório de Microbiologia, Instituto Federal de Educação, Ciência e Tecnologia do Rio de Janeiro (IFRJ), Rio de Janeiro, Brazil.
[2] Laboratório de Higiene e Microbiologia de Alimentos, Faculdade de Farmácia, Universidade Federal Fluminense (UFF), Niterói, Brazil.

*Corresponding author: janaina.nascimento@ifrj.edu.br

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ABSTRACT

Most of the culture media used in bacterial growth is composed of complex ingredients, increasing the value of the product. This makes its acquisition unavailable by educational institutions without sufficient funding, making even more difficult the practical teaching of microbiology. Therefore, the development of an alternative medium of simple composition and low-cost becomes necessary. This work aimed to use texturized soy protein (TSP) as a low cost culture medium that allows the bacterial growth. For the composition of the broths, concentrations between 0.5% and 10% were prepared. Thirty-eight bacteria, including important pathogens associated with food, were inoculated and the concentration of 7.5% TSP allowed the growth of 100% of the tested bacteria, with a production cost of approximately 86% and 68% lower than tryptic soy broth and agar, respectively. This work demonstrates that the use of a culture medium of easy acquisition and low cost is feasible and has good results.

Keywords: low-cost culture medium, textured soy protein, bacterial growth, practical teaching of Microbiology

INTRODUCTION

Soybeans are important source of low cost protein and have high nutritional value, besides their health benefits (**Vagadia *et al*., 2017**). In Brazil, soybean is the main crops both in area and in production. Only at the end of 2019, Brazil exported a record 10.3 million tons of soybeans (**Cattelan and Dall'Agnol, 2018**; **USDA/FAS, 2019**).
Different products can be obtained from soybeans, as miso, soymilk, soy cheese, soy yogurt, soy sauce, tofu, textured vegetable protein and textured soy protein (**Jayachandran and Xu, 2019**). Textured vegetable proteins are obtained from edible protein sources and are characterized by structural integrity and texture that resist the procedures used in preparing food for consumption. The textured soy protein (TSP) is generally obtained by high temperature extrusion of defatted soy flour (**Wu *et al*., 2018**) and its main application, without a doubt, is in human food, in substitution to ground meat, but it can also be used in animal feed (**Stein *et al*., 2008; Jovanovic *et al*., 2019**).
Many culture media used for the growth of microorganisms also have some soy derivatives in their composition, but in general, they are made up of complex ingredients, which increase the value of the product.
Taking into account the high nutritional content of TSP and its relatively low cost for the domestic market, this work aimed to develop a non-selective culture medium based on TSP in order to enable its use for the growth of various types of bacteria in educational and research institutions with limited resources, validating their effectiveness in the growth of several species of bacteria.

MATERIAL AND METHODS

Formulation of TSP medium

Four TSP brands have been acquired in local markets. For the composition of the broths, the samples were ground in an electric blender and different concentrations (0.5%, 1.0%, 2.5%, 5%, 7.5% and 10% [w/v]) were prepared in distilled water, filtered on filter paper to remove debris and autoclaved. For the composition of the solid medium, 1.5% [w/v] agar-agar was added.

Evaluation of bacterial growth on TSP agar

For the initial evaluation of the most promising formulation, fifteen species of Gram-negative and Gram-positive bacteria isolated from food, previously identified by mass spectrometry (MALDI-TOF) and with different levels of nutritional requirements, were subjected to growth in the six formulations of TSP. Important food pathogens such as *Bacillus cereus*, *Escherichia coli* and *Staphylococcus aureus* were included. The bacteria were grown on tryptic soy agar (Casoy agar, Himedia, São Paulo, Brazil) and diluted in 0.85% [w/v] saline solution, to a concentration of approximately 1.5 x 10^8 CFU.ml^{-1}. Twenty microliters of each suspension were inoculated on the surface of the solid media and the plates were incubated at 37 °C for 18 h.

Quantification of bacterial growth in TSP broth

With the formulation that provides the best results (the lowest concentration used that allowed the growth of the largest number of bacteria) in the previous step, a total of 38 bacteria belonging to different species were tested for growth. Tryptic soy broth (Casoy broth) was used as a positive control. The bacteria were grown on Casoy agar at 37 °C for 18 h and inoculated in 0.85% [w/v] saline solution, to a concentration of approximately 1.5 x 10^8 CFU.ml^{-1}. Aliquots of this dilution (corresponding to 10^6 CFU.ml^{-1}) were inoculated in tubes with TSP broth and Casoy broth, which were then incubated at 37 °C for 18 h. After this period, bacterial quantification was performed on Casoy agar, to compare the bacterial growth in the two culture media.

Statistical analysis

Statistical analysis of the mean values of CFU.ml^{-1} obtained from bacterial growth in TSP and Casoy media was performed by unpaired two-tailed t-test (GraphPad QuickCalcs Web software), considering $p \leq 0.05$ as statistically significant.

Economic evaluation

A price research of TSP medium, Casoy medium and agar-agar (necessary for making TSP agar) of at least five different brands and marketed by several companies in Brazil was carried out in order to calculate the average production value of the TSP medium.

RESULTS AND DISCUSSION

Some works described in the literature have also tried to develop low-cost culture media, using different substrates. Adesemoye & Adedire (2005), for example, used different cereals extracts (corn, millet and sorghum) as basal medium for the growth of fungi. In a study conducted in India, an alternative culture medium for bacterial growth from fruit and vegetable remains was developed (Jadhav *et al*., 2018). Recently, Gabunia *et al*. (2019) verify that corn husk extract can be used for the growth of *Staphylococcus aureus* and *Escherichia coli*. In another study, the main food pathogens such *as S. aureus, E. coli, Bacillus cereus, Pseudomonas aeruginosa* and some types of molds showed satisfactory growth in culture media developed from vegetables such as lentils, chickpeas and peas. (Sharref, 2019). Uthayasooriyan et al. (2016), found that *Klebsiella* sp. showed more abundant growth in a culture medium developed from chickpeas than in commercial nutrient agar. The authors also used soy flour to prepare a culture medium, but the results were more promissory with the fungi then the bacteria tested.
However, the present work appears to be the first to use textured soy protein as the only source of nutrient for the composition of a low-cost culture medium. The TSP agar formulation containing concentrations of 7.5% and 10% TSP proved to be more promising, with the growth of all of the tested bacteria, as shown in

Table 1. Some species also showed a proteolysis halo in the 10% TSP medium, a characteristic of the culture medium to be studied in further works. It is worth mentioning that there was no difference in bacterial growth in the 4 brands of TSP tested. The appearance of TSP is shown in Figure 1.

Table 1 Initial assessment of the bacterial growth at different concentrations of TSP agar

Bacteria tested	Concentration of textured soy protein on TSP agar						Control
	0.5%	1.0%	2.5%	5.0%	7.5%	10%	(Casoy agar)
Gram-positive							
Bacillus cereus	++	++	+	+	++	++*	++
Micrococcus luteus	++	+	+	++	+	++*	++
Staphylococcus aureus	++	+	+	++	++	++	++
Gram-negative							
Enterobacter absuriae	-	+	++	+	++	++	++
Escherichia coli	++	+	+	+	++	++*	++
Hafnia alvei	-	+	+	+	+	++	++
Klebsiella ornythinolytica	++	++	++	++	++	++	++
Klebsiella pneumoniae	-	++	++	++	++	++	++
Leclercia adecarboxylata	+	+	-	-	+	+	+
Pantoea agglomerans	++	+	++	++	++	++	++
Serratia liquefaciens	++	++	++	++	+	++	++
Serratia marcescens	++	+	++	++	+	++*	++
Stenotrophomonas maltophilia	+	-	+	+	++	++	+

Legend: -: no growth; +: sparse growth (isolated colonies); ++: abundant growth; *: presence of a proteolysis halo.

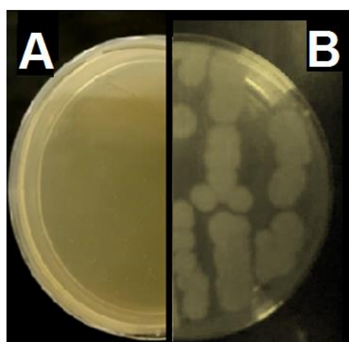

Figure 1: Appearance of 7.5% TSP agar. A: non-inoculated; B: inoculated with *Bacillus* sp.

Once the 7.5% concentration was selected as ideal, 38 isolates from different food matrices were subjected to growth in 7.5% TSP broth for 24h. All showed good to excellent growth, similar to the control in Casoy broth, with counts between 1.1 x 10^9 and 5.1 x 10^{12} CFU.ml^{-1} after the incubation period, as shown in Table 2. Statistical analysis of the mean values of CFU.ml^{-1} obtained from bacterial growth in TSP and Casoy media showed that the two-tailed P value was 0.9031. By conventional criteria, this difference was considered to be not statistically significant, showing the potential of using the TSP broth to replace the Casoy broth.

Table 2 Quantification of different bacteria isolated from food in 7.5% TSP broth and in Casoy broth after incubation for 24 h

Genera	Species	Counts* (CFU.mL^{-1}) after 18h incubation	
		TSP Broth	Control (Casoy broth)
Acinetobacter [1]	*A. bereziniae*	1,1 x 10^{10}	5,0 x 10^{12}
	A.guillouiae	5,0 x 10^{12}	9,5 x 10^{10}
	A.ursingii	3,4 x 10^{10}	1,6 x 10^{11}
Enterobacter [2]	*E. asburiae*	1,0 x 10^{10}	5,0 x 10^{10}
	E. cloacae	5,0 x 10^{10}	5,0 x 10^{10}
	E. hormaechei	1,5 x 10^{10}	6,0 x 10^{9}
	E. kobei	8,6 x 10^{11}	5,0 x 10^{12}
Escherichia [3]	*E. coli*	5,0 x 10^{12}	5,0 x 10^{10}
Klebsiella [2]	*K. oxytoca*	4,8 x 10^{11}	2,9 x 10^{10}
	K. pneumoniae	5,0 x 10^{12}	1,6 x 10^{10}
	K. varicola	9,6 x 10^{10}	5,0 x 10^{12}
Leclercia [2]	*L . adecarboxylata*	3,6 x 10^{11}	8,9 x 10^{10}
Pantoea [2]	*P. agglomerans*	2,2 x 10^{11}	9, 0 x 10^{9}
Pseudomonas [4]	*P. chloraphis*	9,6 x 10^{11}	5,0 x 10^{12}
	P. extremorientalis	9,5 x 10^{11}	5,0 x 10^{12}
	P. koreensis	8,3 x 10^{10}	1,4 x 10^{11}
	P. libanensis	5,0 x 10^{12}	9,0 x 10^{11}
	P. mosseli	5,0 x 10^{12}	8,6 x 10^{10}
	P. nitroreducens	8,7 x 10^{10}	5,0 x 10^{12}
	P. plecoglossicida	2,2 x 10^{11}	2,6x 10^{10}
	P. synxantha	6,5 x 10^{11}	1,0 x 10^{10}
	P. tolaasii	1,5 x 10^{10}	6,7 x 10^{11}
	P. vancouverensis	5,4 x 10^{10}	5,1 x 10^{12}
Raoutella [2]	*R. ornithinolytica*	1,0 x 10^{11}	1,0 x 10^{9}
Serratia [2]	*S. liquefaciens*	5,0 x 10^{10}	1,1 x 10^{9}
Stenotrophomonas [2]	*S. maltophilia*	5,0 x 10^{12}	8,3 x 10^{11}
Bacillus [3,5]	*Bacillus* sp. A	1,3 x 10^{10}	2,0 x 10^{10}
	Bacillus sp. B	1,0 x 10^{10}	1,3 x 10^{11}
	Bacillus sp. C	3,5 x 10^{10}	4,0 x 10^{11}
	Bacillus sp. D	3,2 x 10^{10}	4,5 x 10^{10}
	B. cereus	3,7 x 10^{11}	5,0 x 10^{10}
	B. megaterium	5,0 x 10^{10}	5,0 x 10^{10}
	B. stearothermophilus	4,2 x 10^{11}	5,0 x 10^{10}
Staphylococcus [5]	*Staphylococcus* sp. A	4,2 x 10^{11}	2,5 x 10^{10}
	Staphylococcus sp. B	5,8 x 10^{11}	6,5 x 10^{10}
	Staphylococcus sp. C	5,0 x 10^{10}	5,0 x 10^{10}
	Staphylococcus sp. D	4,2 x 10^{10}	1,3 x 10^{11}
	Staphylococcus sp. E	5,0 x 10^{10}	3,0 x 10^{10}

Legend: *The values represent the average of two independent experiments. Sources of isolates: [1]Ramos & Nascimento, 2019; [2]Ramos, 2019; [3]Bank of bacteria from the Microbiology Laboratory of IFRJ; [4]Ramos & Nascimento, 2020; [5]Oliveira et al., 2012.

In developing countries, research and classes involving microorganisms are often not carried out due to the scarcity of resources for the acquisition of the necessary inputs (**Uthayasooriyan *et al*., 2016**). The value of conventional culture media, both in agar and in casoy broth, is extremely high because it has complex ingredients. For this reason, a price survey was carried out to verify whether the TSP would be a good substitute for conventional culture media with a more accessible value. It was found that the production cost of TSP broth in Brazil is, on average, about 88% less than Casoy broth and 69% less than Casoy agar, respectively, as shown in Table 3.

Table 3 Comparison of production costs between Casoy and TSP media

Value per gram (USD[1])		Approximate average value needed for prepare 1 liter of culture medium (USD)		Cost difference
Casoy broth	0.114 ± 0.019	Casoy broth (30g/L)	3.42	87.7%
Textured soy peptone	0.006 ± 0.002	7.5% TSP broth (75g/L)	0.42	
Casoy agar	0.183 ± 0.067	Casoy agar (45g/L)	8.23	68.2%
agar-agar	0.147 ± 0.024	7.5% TSP agar[2]	2.62	

Legend: [1]The values in dollars were quoted in March 2020, from the values in reais (Brazilian currency). [2] TSP broth 7.5% plus 1.5% (w/v) agar.

CONCLUSION

The TSP culture medium, at a concentration of 7.5%, presents efficiency comparable to the growth patterns of commercial media, at a lower cost, being more accessible mainly to educational institutions that do not have large resources for Microbiology and small research laboratories, in activities that aim only at bacterial growth. The use of more bacteria isolated from food, especially Gram-positive ones, and statistical analysis of the results are being carried out in order to expand and improve the data on the potential use of the TSP medium.

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BACTERIAL EMPIRE
2020, VOL. 3, NO. 4, 77-80

BIODIVERSITY OF SPECIES AND ANTIMICROBIAL RESISTANCE OF BOVINE MILK WITH CLINICAL AND SUBCLINICAL MASTITIS

V. Mazurenko[1, 2], *D. Dreval*[1], *I. Sobko*[1]

Address (es):
[1] "Veterinary Diagnostic Center", LTD Kiev, Ukraine.
[2] NSC "Institute of Biology and Medicine" of the National Taras Shevchenko University of Kyiv, Ukraine.

*Corresponding author: mazurenkovictoriaa@gmail.com

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ABSTRACT

This article was discussed the problems biodiversity of cow mastitis. The purpose of the work was to conduct a statistical analysis of microbiological data milk samples from cows with mastitis in order to ensure the targeted use of antibiotics on Ukrainian farms. Also, the article reveals the problems of the aseptic sampling technique for analysis, which complicates their identification. The obtained milk samples from 20 farm of Ukraine were examined using microbiology methods. As a result, 41% of all isolated bacteria were contagious mastitis agents: 15% of *Staphylococcus aureus* and 26% of *Streptococcus agalactiae*, and 59% were environmental mastitis agents. The most bacteria refer to Gram (+) microflora, namely streptococci (22.5% - *Streptococcus spp*. (*S.agalactiae* not include) and coagulase-negative stain (CNS) of staphylococci (14% - *Staphylococcus spp.* CNS). 18.5% of all isolated isolates are identified as Gram (-) microflora: for by Gram (-) microflora: 11% - *E.coli*, 6% - *Pasteurella spp*., 1.5% - *Proteus spp*. Mastitis caused by fungi (yeast), accounted for 1.5% of all diagnosed mastitis. Most of the isolates were sensitive to amoxicillin + clavulanic acid and gentamicin -93, 5%. The smallest number of isolates were sensitive to tylosin- 20,9% and streptomycin- 48,3%. A significant percentage (83.8% - 54.8%) of obtained isolates were sensitive to rifampicin, amoxicillin, bacitracin, cloxacillin, trimethoprim, florfenicol, ampicillin, lincomycin, cephalexin, enrofloxacin, neomycin, penicillin.

Keywords: biological diversity; *Streptococcus agalactiae*; intramammary infection; profile of antibiotics; bacteriological cultivation

INTRODUCTION

The biological diversity of different groups of microorganisms varies greatly on Earth. The literature data indicate a wide variety within the population strain of bacteria, which is an important factor in adapting bacteria to unstable environmental conditions (**Swift et al., 2004**). There is an opinion that the widespread use of antibiotics and disinfectants in agriculture owning to lead to the uncontrolled spread of these resistant microorganisms in ecosystems (**Theuretzbacher et al., 2013**).
Mastitis is a common multifactorial disease of dairy cattle, but its main cause is still the penetration of bacteria into the parenchyma (**Sudhan et al., 2010**).
Contagious agents were shown in previous studies have the ability to latent infection and are the root cause of subclinical inflammation of the mammary gland (**Riekerink et al., 2006**). There are many contagious microorganisms that cause inflammation of the mammary gland, such as *Prototheca spp*., *Corynebacterium bovis*, but the main contagious agents are *Streptococcus agalactiae*, *Staphylococcus aureus* and *Mycoplasma bovis*., with the exception of some mycoplasma infections that may come from other parts of the body and spread on the system level. These three organisms enter the mammary gland through the nipple channel and rapidly spread from cow to cow during milking (**Keefe et al., 1997**).
Environmental mastitis– caused by opportunistic bacteria *Escherichia coli*, *Klebsiella spp*., *Pseudomonas spp*., *Proteus spp*., *Serratia spp*., gram-positive bacilli, yeasts, *Streptococcus spp*. (except *S. agalactiae*), *Staphylococcus spp*. (except *Staph. aureus*), which spread mainly outside the milking parlor (**Hogan at al., 1997**); (**Blum et al., 2017**).
Cultivation of bacteria in milk samples determines the status of infection. Depending on the pathogen, its sensitivity and resistance to antibiotics and the clinical condition of the udder, veterinarians choose the appropriate method of treatment in each particular case (**Dohoo et al., 2011a**). The goal of microbiological study is to identify microorganisms investigated present in milk. The results of bacteriological study make sense in many respects: they provide targeted use of antibiotics, inform about the resistance of pathogens, etc. (**Dohoo et al., 2011b**). The basic requirements for microbiological study are taking milk samples in aseptic conditions and before starting antibiotic therapy. In addition, the point of sampling has a great impact on the results of bacteriological study (**Andersen et al., 2010**).
It is considered that approximately 20-25% of bacteriological studies of acute forms of mastitis have negative results. It is shown, during the analys is of milk from cows with severe cases of clinical mastitis, 70% of cases received negative results of bacteriological examination (**Ganguly et al., 2017**). As a result, microscopic examination of milk precipitate, phagocyte grams of negative bacilli in leukocytes have often been detected. After freezing, thawing and cultivation, in some cases, it was possible to isolate the pathogen (**Czyżak-Runowska et al., 2018**).
Furthermore, it is noted that SCC can be used as a biomarker for prompt diagnosis of both clinical and subclinical mastitis as well as to ascertain the effectiveness of the therapeutic regimen. This will ensure early detection of subclinical mastitis and will enable successful implementation of mastitis control programmes to ensure quality milk production (**Das et al., 2018**).
The purpose of the work was to study the variety of pathogenic and conditionally pathogenic flora found in milk of cows, as well as its antibiotic sensitivity.
To achieve the goal, the following tasks were set:

1. Determine the pathogens that cause mastitis within Ukraine.
2. Analyze antibiotic sensitivity and resistance of isolated mastitis agents.

MATERIALS AND METHODS

The obtained milk samples from 20 farm of Ukraine were examined using the bacteriological method. The isolation of aerobic bacteria from the milk samples under study was used blood agar (for isolation, identification and determine the type of hemolysis) produced by BioMerieuxtm (France). For pre-identification and selective isolation, McConkey Agar (for isolation Coliforms, E.coli), Mannitol Salt Agar (MSA) (for use as a selective and differential medium for the isolation of pathogenic staphylococci.), Edwards Agar (for the rapid isolation of Streptococcus agalactiae and other streptococci), Saburo Agar (for the cultivation of yeasts, moulds) manufactured by Himedia[tm] (India) and Biolife[tm] (Italy) were used. Bacterial staining was done by Gram's Method. Identification of isolated bacterial isolates was carried out using commercial test systems API 20E BioMerieux[tm] (France) and STREPTOtest [16] ErbaLachematm (Czech Republic). Culture media and commercial tests systems were cultivated 37±1ºC in incubator, 18±2h in an aerobic environment.
Antibiotic sensitivity of the isolated isolates was identified using the disc in vitro diffusion method on the Mühler-Hinton agar with the use of standard commercial disks Amoxicillin-25mcg/disc, Amoxicillin+ClavulanicAcid-20mcg/disc+10mcg/disc, Gentamicin-10mcg/disc, Enrofloxacin-10 mcg/disc, Florfenicol-30 mcg/disc, Streptomycin-10 mcg/disc, Trimethoprim-5 mcg/disc, Ampicillin-10 mcg/disc, Penicillin G-10 units, Tylosin-15 mcg/disc, Neomycin-30 mcg/disc, Lincomycin-15 mcg/disc, Cloxacillin-30 mcg/disc, Rifampicin-5 mcg/disc, Bacitracin-10 mcg/disc, Cephalexin-30 mcg/disc manufactured by Himedia[tm] (India) and Oxoid™ (Florfenicol). Select four to five similar colonies and transfer into suitable broth or saline to obtain turbidity equivalent to 0.5 McFarland barium sulphate standard. The standard ready-made inoculum requires agitation on a vortex mixer before each use. For proper turbidity adjustment, it is helpful to use a white background with contrasting black lines. Inoculate the agar plates directly from the suspension, spreading the inoculums as evenly as possible with sterile swab. Antimicrobial susceptibility test discs are then placed with the aid of flamed tweezers to the inoculated medium. Incubate

the test materials at 37˚ C for 18 hours and then measure the diameters of the inhibition zones surrounding the discs in millimeters.
For statistical processing methods used programs: Microsoft Excel, apiweb[tm] bioMerieux. The interpretation of antibiotic gram results we used some breakpoint tables for interpretation of zone diameters (version 7.1, 2017), developed by the European Committee on Antimicrobial Susceptibility Testing. The European Committee on Antimicrobial Susceptibility Testing. Available at: **http: //www.eucast.org.**

RESULT AND DISCUSSION

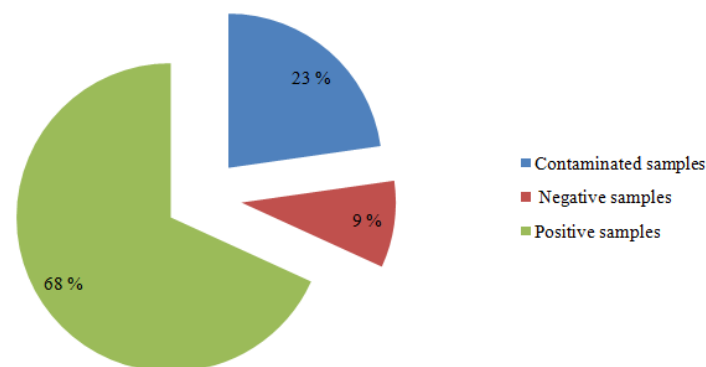

Figure 1 Percentage of milk samples taken for analysis

As seen from the data given in the Fig. 1, the number of milk samples amounted to 92, of which 20 samples contaminated (infected by exogenous microflora), which is 23% of the total number of samples. This is a fairly large percentage, which indicates the contravention of aseptic technology and the neglect of the rules for sampling milk for examination. In most cases, if more than 2 or 3 different types of microorganisms are plated from a sample of milk with a subclinical form of mastitis, such a sample is considered to be contaminated and is excluded from further examination (**Dohoo et al., 2011a**). This is due to the fact that theoretically any microorganism can cause the infection of the udder (**Dohoo et al. 2011b**) but it is unlikely that mastitis can be caused by more than 2-3 different microorganisms at the same time (**Reyher et al., 2011**).
There was a small percentage (9%) of negative samples, that is, those from which no microflora was allocated. This often happens, especially with the samples taken from cows with a clinical form of mastitis. This may be due to many reasons, the main of which include: significant fluctuations of pH in milk; the presence of a significant number of inflammatory cells and various chemicals that appear in milk during inflammation (enzymes, hormones, cellular debris, etc.) and inhibit bacterial microflora; periodic minor isolation of the agent with milk, or the absence of isolated agent (with mastitis caused by *S. aureus*); aseptic (non-infectious) nature of inflammation; violation of the conditions of storage and transportation of selected samples of milk (violation of the temperature control), etc. (**Degen et al., 2015**).

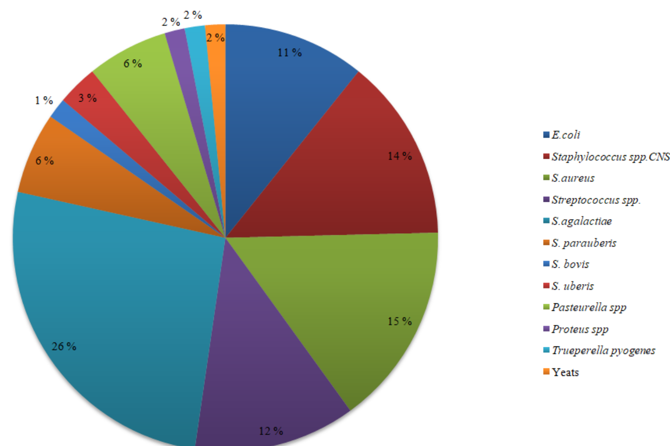

Figure 2 Results of biodiversity of individual milk samples (samples from the affected udder quarter) from cows with clinical and subclinical form of mastitis

Concerning the spectrum of isolated pathogens, 41% of all isolated isolates were contagious mastitis agents: 15% of *S. aureus* and 26% of *S. agalactiae* (pathogenic), and 59% were environmental (conditionally pathogenic) mastitis agents. The most of bacteria refer to Gram (+) microflora that causes environmental mastitis, namely streptococci (22.5% - Streptococcus spp.) and staphylococci (14% - *Staphylococcu spp.*). 18.5% of all isolated isolates are identified as Gram (-) microflora: for by Gram (-) microflora: 11% - *E.coli*, 6% - *Pasteurella spp*., 1.5% - *Proteus spp*. Mastitis caused by fungi (yeast), accounted for 1.5% of all diagnosed mastitis. It was shown in the Fig. 3

Table 1 Distribution of the different number of isolated mastitis agents by their sensitivity to various antibiotics

Active substance of antibiotic	*Staphylococcus aureus*		*Streptococcus agalactiae*		*Escherichia coli*		*Staphylococcus spp (CNS)*		*Streptococcus spp.*		*Streptococcus parauberis*		*Streptococcus bovis*		*Streptococcus uberis*		*Pasteurellla spp.*	
	№	%*	№	%	№	%	№	%	№	%	№	%	№	%	№	%	№	%
Amoxicillin	10	**100**	17	**100**	0	**0**	7	**78**	6	**75**	4	**100**	1	**100**	2	**100**	3	**75**
Amoxicillin+ClavulanicAcid	10	**100**	17	**100**	6	**86**	8	**89**	7	**87.5**	3	**75**	1	**100**	2	**100**	4	**100**
Gentamicin	10	**100**	16	**94**	7	**100**	9	**100**	7	**87.5**	3	**75**	1	**100**	1	**50**	4	**100**
Enrofloxacine	8	**80**	7	**41**	6	**86**	9	**100**	4	**50**	0	**0**	0	**0**	0	**0**	4	**100**
Florfenicol	9	**90**	11	**65**	4	**57**	8	**89**	4	50	3	**75**	0	**0**	0	**0**	3	**75**
Streptomycin	5	**50**	9	**53**	2	**29**	8	**89**	3	**37.5**	0	**0**	0	**0**	0	**0**	3	**75**

Trimethoprim	10	**100**	13	**76**	7	**100**	8	**89**	3	**37.5**	2	**50**	0	**0**	0	**0**	3	**75**
Ampicillin	9	**90**	15	**88**	1	**14**	5	**55.5**	5	**62.5**	3	**75**	1	**100**	0	**0**	3	**75**
Penicilillin	8	**80**	12	**70.5**	0	**0**	4	**44**	4	**50**	3	**75**	0	**0**	0	**0**	3	**75**
Tylosin	3	**30**	5	**29**	0	**0**	1	**11**	1	**12**	0	**0**	0	**0**	0	**0**	3	**75**
Neomycin	9	**90**	10	**59**	0	**0**	9	**100**	3	**37.5**	0	**0**	0	**0**	0	**0**	4	**100**
Lincomycin	9	**90**	16	**94**	0	**0**	8	**89**	2	**25**	2	**50**	0	**0**	0	**0**	4	**100**
Cloxacillin	9	**90**	17	**100**	0	**0**	9	**100**	4	**50**	3	**75**	1	**100**	2	**100**	3	**75**
Rifampicin	10	**100**	17	**100**	0	**0**	9	**100**	6	**75**	4	**100**	0	**0**	2	**100**	4	**100**
Bacitracin	9	**90**	17	**100**	0	**0**	8	**89**	6	**75**	3	**75**	1	**100**	2	**100**	3	**75**
Cephalexin	10	100	14	82	0	0	7	78	3	37.5	2	50	1	100	1	50	3	75
Total of isolate	**10**		**17**		**7**		**9**		**8**		**4**		**1**		**2**		**4**	

Note: *% – percentage of susceptible isolates in relation to the total number of isolates

Table 2 Distribution of the total number of isolated mastitis agents by their sensitivity to various antibiotics

The active substance of antibiotic	Number of sensitive isolates	%*
Amoxicillin+clavulanic acid	58	93,5
Gentamicin	58	93,5
Rifampicin	52	83,8
Amoxicillin	50	80,6
Bacitracin	49	79
Cloxacillin	48	77,4
Trimethoprim	46	74,1
Florfenicol	42	67,7
Ampicillin	42	67,7
Lincomycin	41	66,1
Cephalexin	41	66,1
Enrofloxacine	38	61,2
Neomycin	35	56,4
Penicillin	34	54,8
Streptomycin	30	48,3
Tylosin	13	20,9

Note: *% – percentage of susceptible isolates in relation to the total number of isolates

As seen from the data given in the Tab. 2 and Tab. 3, most of the isolates were sensitive to Amoxicillin + Cl. Acid and Gentamicin -93, 5%. The smallest number of isolates were sensitive to tylosin- 20,9% and streptomycin- 48,3%. A significant percentage (83.8% -54.8%) of obtained isolates were sensitive to: rifampicin, amoxicillin, bacitracin, cloxacillin, trimethoprim, florfenicol, ampicillin, lincomycin, cephalexin, enrofloxacine, neomycin, penicilli.

The sensitivity of *E. coli* isolates is somewhat different. Thus, all 7 isolates of this agent were resistant to amoxicillin, penicillin, tylosin, neomycin, lincomycin, cloxacillin, rifampicin, bacitracin, and cefalexin. 6 out of 7 isolates were resistant to ampicillin and 5 out of 7 to streptomycin. All isolated *E.col* isolates were sensitive to gentamicin and trimetoprim (100%) and a significant percentage (86%) - to amoxicillin + clavulanic acid and enrofloxacin.

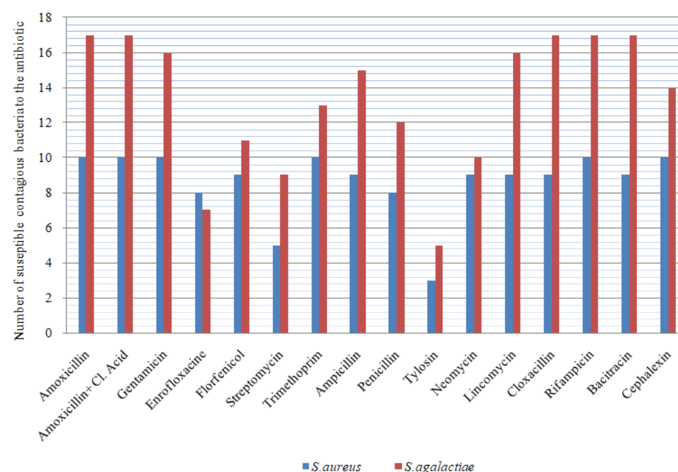

Figure 3 Antibiotic sensitivity of contagious mastitis pathogens (*Streptococcus agalactiae, Staphylococcus aureus*). It is shown in the Fig. 4 antibiotic sensitivity of contagious agents such as *Staph. aureus* and *S. agalactiae* was approximately identical. Most isolates of contagious agents were susceptible to amoxicillin, amoxicillin + clavulanic acid acid, gentamicin, ampicillin, lincomycin, cloxacillin, rifampicin, bacitracin, cephalexin and trimetoprim; and resistant to tylosin and streptomycin.

CONCLUSIONS

A significant percentage of samples (22%) that were submitted for examination were contaminated, suggesting an inappropriate sampling technique and contravention of the conditions of storage and transportation of samples for laboratory testing. In order to prevent contamination of samples, the existing recommendations should be clearly observed. We studied the diversity of biological isolates in milk samples conducted by us; indicate that strains streptococci which account for 48.5% of all diagnosed case is able to primer case the cause of mastitis. Among Gram-negative pathogens causing mastitis *E. coli* constitute a majority, but in relation to the total number of diagnosed mastitis the percentage of mastitis caused by *E. coli* amounted to only 11%. With regard to the findings, the antibiotic of choice is amoxicillin + cl. acid and gentamicin. The antibiotic of the last choice was tylosin.

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BACTERIAL EMPIRE

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SCICELL

ASSESSMENT OF THE ANTIBIOTIC RESISTANCE OF BACTERIAL ISOLATES RECOVERED FROM DOG FEED SOLD IN ADO-EKITI METROPOLIS

ATERE Ayowole Victor

Address (es):
Department of Microbiology, Federal University of Technology, Akure, P.M.B 740 Akure, Nigeria.

*Corresponding author: victor_efosa001@yahoo.com

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ABSTRACT

Animal feed has been incriminated in many animal infectious diseases. This study was carried out to investigate and document the bacteriological safety of dog feed sold in Ado-Ekiti Metropolis. Five feed samples were analyzed, the bacterial load, coliform count, total Salmonella count as well as test for campylobacter was carried out on the feed samples. Pure culture of the isolates were subjected to antibiotic susceptibility test using disc diffusion method. The total bacterial count ranged between 4.41 log_{10}CFU/g and 6.36 log_{10}CFU/g. All feed samples had coliform ranging between 2.09 log_{10}CFU/g and 3.93 log_{10}CFU/g. Salmonella was only recovered from feed sample DED. Only sample DDL harbours *Campylobacter* sp. Other bacteria isolated from the feed were *Escherichia coli*, *Bacillus* sp, *Enterobacter* sp, *Klebsiella* sp, *Staphylococcus* sp and *Lactobacillus* sp. The antibiogram showed that nitofuratoin and ciproflocacin had the best activity where 93.10 % (54 out of 58) were susceptible to the two antibiotic. Ampicilin was most resisted with only 36.21 % (21) susceptibility. Thirty one (31) of the isolates showed resistance to 3 or more antibiotic constituting 53.44 % of the entire bacterial isolate. Antibiotic resistance pattern mostly encountered were AMP, AMO, TLY and AMP, AMO, FUR with thirteen (13) isolates each. These results showed that dog feed may not be totally safe for the pets. Based on the type of bacteria isolated and the antibiotic resistance, good manufacturing practices should be ensured by manufactures in other to reduce the rate of contamination.

Keywords: Dogs, *Campylobacter*, *Salmonella*, Antibiotic, Resistance

INTRODUCTION

Animal feeds are so important to the overall productivity of the animal. In general, the composition of animal feed includes mixture of different ingredients that constitute the raw material. The ingredients may include cereals, fat supplements, vitamins, minerals, antioxidants as well as meat meal **(Cabarkapa et al., 2009; Atere et al., 2015a)**.
When feed is contaminated, the quality of the feed is reduced, this contamination often emanates from the ingredients of both plant and animal origin. The quality and quantity of biological contamination is often dependent on the temperature and humidity of the feed and storage environment.
Researchers have showed that the major source of microbial infection in animal is often through the feed consumed, at times, these feeds are contaminated by additives **(Atere, 2016)**. Several animal diseases and syndrome has been traced to the feed, such as diarrhoea, bacillary dysentery, salmonellosis, staphylococcosis, colibacillosis, erysipelas, listeriosis **(Healing and Greenwood, 1991, Xin, 2013)**.
Generally, animal feed has been reported as a source of infection in poultry **(Cabarkapa et al., 2009)**. The toxigenic content of the dog feeds are reported **(Boermans and Leung, 2007)**. A potentially risky factor is the presence of microbial toxins of bacterial and fungal origin in the feed which could lead to food poisoning **(Hussein and Brasel, 2001)**. However, little or no comprehensive information has been reported about the bacteriological contamination and antibiotic susceptibility of the bacteria isolated from dog feed. The bacteriology of the feed can give a clue to the risk feed may constitute, it can also create a public health issue since this can be a source of infection in humans. It is therefore imperative to investigate the bacteriological quality of dog feed sold around Ado-Ekiti metropolis.

AIM AND OBJECTIVE

This research was design to investigate the bacteriological quality of dog feeds by the way of determining the microbial load, the type of bacteria associated with each feed as well as the antibiotic susceptibility of the bacterial isolates.

MATERIALS AND METHOD

The materials used for this research was gotten from pet shops in Ado Ekiti. Triplicate samples of five different feed samples were collected and transported to the microbiology laboratory within two hours of collection.

Determination of moisture content

Five grams of the feed was weighed and dry to a constant weight in a dry oven (DHG-9101-1US Royalcare England). The change in weight divided by the initial weight multiply by 100 was recorded as the moisture content **(AOAC, 2005)**.

Determination of the Bacteriology of the feed

The feed samples were aseptically weighed, 5 gram of each feed sample was homogenized in 45ml of sterile buffer peptone water. A serial dilution of the stock was then carried out using sterile buffered peptone water. The pour plate technique was used where 1 ml of the diluted sample was plated on Nutrient agar, MacConkey and Salmonella-Shigella Agar. All plates were allowed to gel, and inverted then incubated at 37°C for 24h in an incubator (DNP-9022A Royalcare England). The colonies on each of the plates were counted using colony counter. The colonies on the Nutrient agar was recorded as the total bacterial count, the coliforms were determined on MacConkey agar, the total salmonella was determined on Salmonella-Shigella Agar. Preston selective agar was used in testing for the presence of *Campylobacter* sp **(Weese et al., 2005)**.

Identification of bacteria isolates

Pure culture of each isolates were made on nutrient agar. From the pure culture, the cultural characteristics of the colonies were determined. The gram reaction and biochemical testes were also carried out on each of the pure isolates according to the standard as described by **Atere *et al*. (2015b)**. The biochemical characteristics observed were citrate, methyl red voges-proskauer, nitrate, urease, oxidase, coagulase, and catalase.

Antibiotic susceptibility

Muller-Hinton agar was used for the antibiotic susceptibility. The organisms were standardized going by the McFarland standard. While the disk diffusion method was adopted, isolates were inoculated on the agar and the antibiotics disks were introduced. The plate was inverted and incubated in the incubator at a temperature of 37°C for 24 h. The susceptibility and resistance of the bacteria isolates was determined based on the diameter of the zone of inhibition which was compare with the internationally acceptable standard.

RESULTS

The total bacterial count of the feed analyzed ranged between 4.28 log_{10}CFU/g and 6.36 log_{10}CFU/g in feed samples of PED and ERY respectively (Table 1). The highest coliform count was recorded in feed sample DDL with a value of 3.93 log_{10}CFU/g which is significantly higher than all other samples. *Salmonella* sp was not isolated in four of the feeds, the salmonella count of 1.34 log_{10}CFU/g was recorded for feed DED. The moisture content of the feed ranged from 9.16 % to 19.20 %.

Table 1 The moisture content, total bacteria, coliform and *Salmonella* count of dog feed samples

Feed	Total bacterial count (log_{10}CFU/g)	Total coliform count (log_{10}CFU/g)	Total salmonella (log_{10}CFU/g)	Moisture content (%)
DDL	5.68±0.35^{c}	3.93± 0.20^{d}	0	19.2±2.50^{c}
DCO	4.77±0.40^{ab}	3.09±0.40^{c}	0	9.16±0.83^{a}
ERY	6.36±0.50^{d}	2.09±0.30^{a}	0	17.50±2.50^{bc}
PED	4.28±0.10^{a}	2.33±0.15^{ab}	0	11.87±1.49^{ab}
DED	4.41±0.16^{ab}	3.15±0.10^{a}	1.34±0.02	10.00±1.70^{a}

Table 2 showed the distribution of the isolated bacteria in the feed samples. A total of fifty eight (58) bacterial isolates were recovered from all the feeds samples with 8 different bacteria species. *Bacillus* sp was recovered from DDL, DCO, and ERY while *E. coli* was present in all the feed sample analyzed. *Staphylococcus* sp was found in ERY, PED and DED. Only DCO had *Enterobacter* sp. *Campylobacter* sp, was found only in DDL.

Table 2 Number of Bacterial isolates recovered from dog feed samples

Feeds	*E. coli*	*Campylobacter* sp	*Bacillus* sp	*Enterobacter* sp	*Klebsiella* sp	*Lactobacillus* sp	*Staphylococcus* sp	*Salmonella* sp	Total
DDL	6	3	4	-	-	-	-	-	13
DCO	4	-	4	2	2	-	-	-	12
ERY	3	-	2	-	-	6	3	-	14
PED	2	-	-	-	1	-	2	-	5
DED	5	-	-	-	-	-	3	6	14
Total	20	3	10	2	2	6	8	6	58

Table 3 showed the susceptibility of the bacteria isolate to the antibiotic used in this research. The bacterial isolates are most susceptible to nitrofurantoin and ciprofloxacin with 54 of the bacterial isolates being susceptible constituting 93.10% of the total isolates. The least active antibiotic was ampicillin with only 36.21 % (21) susceptible.

Table 3 Antibiotic susceptibility profile of bacterial isolates recovered from dog feed samples

		CEF	CEZ	AMO	OFL	TLY	CIP	ENR	NIT	FUR	GEN	AMP
DDL	*Bacillus* sp n=4	4	3	3	4	1	4	2	4	1	3	1
	Campylobacter sp n=3	2	2	1	2	2	2	1	2	1	3	2
	E. coli n=6	4	5	1	5	5	5	4	6	4	6	2
DCO	*Enterobacter* sp n=2	2	2	2	2	1	2	2	2	2	2	1
	E. coli n=4	3	4	2	4	2	4	2	4	1	3	1
	Bacillus sp n=4	4	4	3	4	2	4	4	4	3	4	3
	Klebsiella sp n=2	2	2	2	2	1	2	2	2	2	2	1
ERY	*E. coli* n=3	2	1	1	2	1	3	1	2	1	2	0
	Lactobacillus sp n=6	6	6	5	6	5	6	5	6	4	6	3
	Bacillus sp n=2	2	1	0	2	2	2	1	2	2	2	1
	Staphylococcus sp n=3	1	3	3	3	1	3	3	3	3	3	2
PED	*Klebsiella* sp n=1	1	1	0	0	0	1	1	1	0	1	0
	Staphylococcus sp n=2	2	2	0	2	0	2	2	2	0	0	0
	E. coli n=2	2	2	2	2	2	2	2	2	0	2	0
DED	*Salmonella* sp n=6	5	4	3	5	4	6	2	5	3	5	2
	E. coli n=5	4	4	2	5	3	4	3	4	2	4	1
	Staphylococcus sp n=3	2	3	1	3	1	2	1	3	3	2	1
Total	58	48	49	31	53	33	54	38	54	32	50	21

Key: Ceftazidime (CEZ) and Cefuroxime (CEF) Amoxicillin(AMO), Ofloxacin (OFL), Tylosin (TLY), Ciprofloxacin (CIP), Enrofloxacin (ENR), Nitrofurantoin (NIT), Furasol (FUR), Gentamicin (GEN), Ampicillin (AMP).

The antibiotic resistance pattern AMP, AMO, TLY and AMP, AMO, FUR were the most encountered resistance pattern with 13 bacterial isolates each displaying such resistance pattern. NIT, GEN, AMP and CIP AMP TLY resistance patterns were least encountered with one isolate each displaying this antibiotic resistance pattern (table 4). Table 5 showed the distribution of bacterial isolates showing resistance to 3 or more antibiotics. A total of 31 out of 58 bacterial isolates recovered from dog feeds showed resistance to 3 or more antibiotics.

Table 4 Antibiotic resistance pattern of Bacterial isolates recovered from dog feed samples

	DDL			DCO				ERY				PED			DED			
Resistance pattern	*Bacillus* sp	*Campylobacter* sp	*E.coli*	*Enterobacter* sp	*E. coli*	*Bacillus* sp	*Klebsiella* sp	*E. coli*	*Lactobacillus* sp	*Bacillus* sp	*Staphylococcus* sp	*Klebsiella* sp	*Staphylococcus* sp	*E. coli*	*Salmonella* sp	*E. coli*	*Staphylococcus* sp	Total
AMP, AMO, TLY	1	1	1	0	1	1	0	1	0	0	0	1	2	0	2	1	1	13
AMP, AMO, FUR	1	1	0	0	1	1	0	1	1	0	0	1	2	0	2	2	0	13
CEF, CEZ, OFL	0	0	1	0	0	0	0	1	0	0	0	0	0	0	0	1	0	3
NIT, GEN, AMP	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1
ENR, AMP, AMO, FUR	0	1	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	3
CIP, AMP, TLY	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1

Key: Ceftazidime (CEZ) and Cefuroxime (CEF) Amoxicillin(AMO), Ofloxacin (OFL), Tylosin (TLY), Ciprofloxacin (CIP), Enrofloxacin (ENR), Nitrofurantoin (NIT), Furasol (FUR), Gentamicin (GEN), Ampicillin (AMP).

Table 5 Bacterial isolates recovered from dog feeds showing resistance to 3 or more antibiotics

Feed	*E. coli*	*Campylobacter* sp	*Bacillus* sp	*Enterobacter* sp	*Klebsiella* sp	*Lactobacillus* sp	*Staphylococcus* sp	*Salmonella* sp	Total
DDL	3	2	3	-	-	-	-	-	8
DCO	3	-	0	0	0	-	-	-	3
ERY	3	-	1	-	-	1	1	-	6
PED	0	-	-	-	1	-	2	-	3
DED	4	-	-	-	-	-	3	4	11
Total	13	2	4	0	1	1	6	4	31

DISCUSSION

Foods have been recognized as the major health determinant, what you eat determined your wellbeing. Animal feed has been reported as a source of microbes in farmed animals and poultry **(Weese et al., 2005; Atere et al., 2015a)**. This may also be true of pets. The bacterial load and the type of bacteria present in the feed samples analyzed indicated that the feeds might not be totally safe for the animals. Isolating *Campylobacter* sp in one of the feed sample is an indication that such a food is so unfit. The bacteria recovered is an indication of potential hazard to the animals. This study revealed that eight (8) bacterial species were isolated from these feeds. The occurrence of these bacteria in the feed and direct consumption of bacterial contaminated feed or their toxins by such animal may constitute a public health issue **(Fraizer and Westhoff, 1978)**. Animal feeds are rich in nutrients, this encourages the proliferation of microorganism and when the environmental/storage conditions such as moisture increased, the growth of these bacteria are pronounced **(Atere et al., 2015)**.

In a similar investigation carried out by **Weese *et al*. (2005)**, *E. coli, Salmonella* sp *Staphylococcus* sp and *Clostridium* sp were isolated. Meanwhile there was no *Clostridium* sp isolated in this research, a report of coliform present in all the samples analyzed is also similar to what is observed in this research where *E.coli* was isolated in all the feed samples **(Weese et al., 2005)**. **Nemser et al. (2014)** reported that *Salmonella, Listeria* and *E. coli* are often isolated in pet food, the bacteria load and the type of bacteria isolated from feed can tell more about the safety of the feed.

In a previous research of **Atere et al. (2015a)**, it was reported that the presence of coliforms in poultry feed may have resulted from feacal or environmental contamination. This might also be true of what is observed in this study where the coliform level of three of the feeds are higher than what is recommended by Canadian food inspection agency where the maximum level of coliform should be less or equal to 1000 CFU/g **(Fraizer and Westhoff, 1978)**. Coliform count is always seen as index of sanitation. The increase above this level in this feed may suggest that good manufacturing practices are not being stocked to, or may have resulted from improper handling.

Isolating *Salmonella* sp in one of the feed is of concern. This is because salmonella is a pathogen of many farm animals including pets like dog and cats. There are indications that there could be zoonosis through direct contact or through environmental contamination within the house hold **(Atere et al., 2015b)**. The presence of *Campylobacter* sp in one of five feeds is of concern, this is because it has been responsible for food infection in humans **(Brieseman, 1990)**. Meanwhile *Campylobacter* sp is one of the recognized enteropathogen of dogs and cats, where contact with these pets has been reported as the means of transmission of *Campylobacter* sp to human population **(Brieseman, 1990)**.

The presence of *Staphylococcus* sp in three of the feed samples may have resulted from human source, possibly during dispensing, since these are normal flora of human body. It should be recalled that *Staphylococcus aureus* and *Salmonella* sp are capable of producing acute and chronic infection in all or most type of animal **(Mallinson, 1984)**. Therefore, the effect these bacteria could have on dogs should not be underestimated.

The antibiotic susceptibility of the bacterial isolate can also be of great public health concern. Though the isolates are well susceptible to the antibiotic used in this research when compared with the susceptibility of bacteria isolates from poultry feed **(Atere et al., 2015a)**. In research of **Atere et al. (2018)**, it was reported that the bacterial isolates from dog are less resistant to antibiotic when compare to isolates recovered from poultry. The reason for the increased susceptibility of bacterial isolates recovered from the dog feed when compared with that of poultry may be related to what **Atere et al. (2018)** earlier reported as the sub-therapeutic addition of antibiotic to poultry feeds. Nevertheless, this study showed that some of the bacteria showed multiple resistance, it is of great importance to acknowledge that some of the bacteria multiple resistance strain can find human population, through a trend of being infectious in the pets that feeds on them and through human contact with the pets finds its way into human population.

CONCLUSION

There are potential risks attached to the pet feeds, the type of bacteria found in the feed, the load of the bacteria and the antibiotic resistance of these organisms are of public health concern. The storage condition and the moisture content can also encourage the growth of bacteria. Chemical amendment, heat treatment, careful sourcing for raw materials and good manufacturing practices can go a very long way in reducing bacterial infestation of the feed thereby, improving the safety of the feed.

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MICROBIOLOGICAL QUALITY ANALYSIS OF FERMENTED AND UHT MILK SAMPLES COLLECTED FROM DIFFERENT LOCATIONS IN INDONESIA

Anggun Hari Kusumawati[*1], Neni Sri Gunarti[2], Maya Arfania[3], Dedy Frianto[4], Dadan Ridwanuloh[5], Ova Shofa Mudrofah[6]*

Address (es):
[1]Buana Perjuangan Karawang University, Faculty of Pharmacy, St.HS.Ronggowaluyo, Karawang 41371, Indonesia, +6281222821393.

*Corresponding author: anggunhari@ubpkarawang.ac.id

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ABSTRACT

Diarrhea can occur due to food and beverage poisoning, with the highest cause being caused by infection with various bacteria, viruses, or parasites. Bacteria that can cause this disease are Escherichia coli bacteria which are known as good bacteria in the digestive tract. But the reality is that in microbiology not all types of Escherichia coli are good bacteria. Aim to find out the content and number of Escherichia coli bacteria colonies in UHT milk brands A and B as well as brand x yogurt products. Identification of Escherichia coli bacteria by the IMVIC Test method (Indole, Methyl-Red (MR) test, Voges Proskauer (VP), and Citrate), TPC (Total Plate Count), bacterial staining and microscope observation. Negative and positive results were obtained in the indole test and the methyl-red test was characterized by the formation of a red ring at the top for positive results and a yellow ring at the top for negative results, as well as negative results obtained in the Voges Proskauer test and the citrate test. Then for the results of gram staining and microscope testing the bacterial morphology was not seen. For the calculation of colonies, 45 colonies of sample A, 60 colonies of sample B, and 38 colonies of sample X. Samples containing Escherichia coli are contained in sample A of UHT milk and sample X of yogurt products and microbial contamination in samples according to SNI 2009.

Keywords: *Eschericia coli*, UHT Milk, Fermented Milk, IMVIC, TPC, yogurt

INTRODUCTION

Diarrhea can occur due to food and beverage poisoning, with the highest cause being the result of infection with various bacteria, viruses, or parasites. Bacteria that can cause this disease are Escherichia coli bacteria which are known as good bacteria in the digestive tract. Bacteria Escherichia coli is a species of bacteria with natural habitats in the digestive tract of humans and animals. But in reality in microbiology, not all types of Escherichia coli are good bacteria **(Delmas, 2015**). Milk is a special food because of its delicacy and balanced composition. Milk contains substances needed by the body such as protein, fat, carbohydrates, minerals, and vitamins. UHT milk is one type of milk that has been processed. While fermented milk is milk with the addition of good bacteria needed by the body in the digestive system **(Delmas, 2011)**. The pollution that occurs in milk is caused by cows, unclean tools and unclean storage areas, dust, air, flies, and poor handling of personnel. After being released from cows, the content of microorganisms in milk is a function of age that determines the level of development of natural flora, the handling of milk that determines the types of organisms carried, and the storage temperature that determines the breeding speed of all types of organisms **(Dumalisile *et al.*, 2005).** High nutritional substances found in milk are good media for microbial growth. Microbial growth causes milk can not stand stored for long and is easily damaged. One way to preserve milk is fermentation treatment (Delmas, 2016). Fresh liquid milk which is widely used is UHT (Ultra High Temperature) milk. UHT milk is a milk product that is obtained by sterilizing milk at a minimum temperature of 135°C for 2 seconds, without the addition of permitted food ingredients and aseptically packaged. This type of milk is usually packaged in a cup or glass with a variety of flavors. UHT milk is also packaged using cardboard boxes or shaped pads ***(Fischer et a*l., 2011**). UHT milk and fermented milk are easily found by the public, both in small shops and large stores because these products are sold in the form of ready to drink packaging **(Widodo *et al*., 2017**). As a liquid beverage packaging product, UHT milk consumption tends to be more desirable than pasteurized milk. While fermented milk is also a product that is in demand because of its usefulness to the digestive system. So for the selection of pasteurized milk products tend to be less desirable because of constraints on the distribution channel **(Omara et al., 2018).** Pasteurized milk which requires the presence of a cold chain (cooling lane), tends to be durable and easily damaged. Bacterial pollution that occurs in liquid milk packaging that is after the packaging is opened. This can cause digestive disorders due to the influence of microorganisms that grow in the milk and some infection conditions such as urinary tract infections in children and infections of the digestive tract **(Dumalisile *et al*., 2005).**

MATERIAL AND METHODS

Materials

The materials used in this study were UHT milk (samples A and B) and fermented milk (sample X) obtained from supermarkets in the Karawang area. Bacterial growth media are NA (Nutrient Agar), SIM (Sulfide-Indole-Motility), and MR-VP (Methyl Red-Voges Proskauer). The reagents used are Kovac reagents, methyl red reagents. Gram staining is the crystal violet, 96% alcohol, safranin, and aquadest.

Methods

Total Plate Count (TPC)

At Each Sample 1 mL is taken, then diluted using distilled water until dilution to 10^{-6}. Furthermore, From Each Sample 10^{-6} Dilution, 1 mL is taken to be inserted into a sterile petri dish, then poured nutrient agar (NA) liquid media and homogenized by sliding horizontal plates or forming the number eight and allowed to freeze. incubation on milk 37ºC for 24 hours and all colonies that grow are counted as TPC.

Biochemical Test

From the dilution tube, make a scratch on the nutrient agar (na) media, and incubate at 37ºC for 18-24 hours. observe the murky white colonies of NA.

Indol Test

Colonies from na media were inoculated in a sim-filled tube and incubated at 35ºC for 24 hours ± 2 hours. then add a few drops of the kovac reagent until a red ring appears on the top layer for positive results and yellow rings for negative results.

Methyl Red (MR) Test

Colonies from na media were inoculated in tubes containing 10 ml mr-vp and inoculated at 35ºc for 48 hours ± 2 hours. then add 2-5 drops of the mr indicator to the tube. observe the presence of red for positive results and yellow for negative results.

Voges Prokauer Test (VP)

Pure bacterial isolate inoculated on mr-vp media and incubated for 24 hours at room temperature. Add 0.6 ml of 5% alpha naphtol solution followed by 0.2 mL of 40% KOH. observe the color change for 30 minutes.

Citrate Test

Inoculation of pure bacterial isolate by zig-zag scratch using ose or using inoculation needle on simmons citrate media to tilt, then incubate for 24 hours at room temperature. observe the color change from green to blue.

Gram Staining

In NA, take a murky white colony with ose. then place it on the glass preparation, fix it on the fire bypassing the glass preparation on the fire twice. drop gentian violet until the entire circle is covered, wait for 5 minutes. Clean over running water. then drop the lugol and wait for 1 minute. Clean again above running water. drop alcohol on the entire surface until no color wears off again. Clean again above running water. Drop safranin and wait for 2 minutes. clean again above running water. Dry preparations on a tissue.

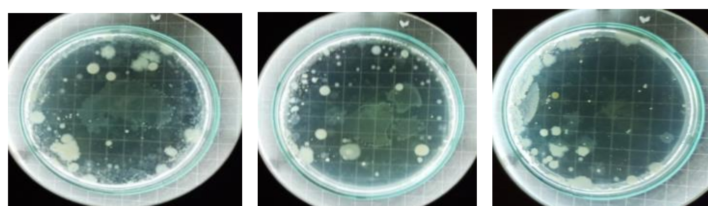
Microscopic examination

The first drop of immersion oil. then check the preparations under the microscope of the smallest magnification first. After finding the colony lay, change the magnification up to 100 times. The appropriate form of escherichia coli is red, short trunk shape, and a single colony.

RESULTS AND DISCUSSION

Number of UHT Milk and Fermented Milk Colonies

Testing the number of colonies is done by using the cup calculation method with a 10-6 dilution. In the calculation of the number of colonies test results obtained are shown in Figure 1 with calculations using the cup calculation method.



Sample A	Sample B	Sample X
45 colonies	60 colonies	38 colonies

Figure 1 Calculation of the number of colonies in dilutions 10^{-6}

From the data above sample X which is a sample of fermented milk has the lowest number of colonies compared to samples A and B which are UHT milk samples. This can happen because the level of dilution is carried out low so that the growth of microorganisms becomes very numerous and difficult to do calculations, whereas for samples A and B the level of dilution is carried out high **(Caplan & Barbano, 2013)**. For samples A and B which are flavorful and non-flavored UHT milk, the results of the calculation of the number of colonies are in accordance with SNI provisions in 2009 concerning the maximum limit of microbial contamination, namely for flavored and non-flavored UHT milk <10 colonies / 0.1 mL or 100 colonies / 1 mL, while for sample X which is fermented milk is also in accordance with the provisions of SNI in 2009 concerning the maximum limit of microbial contamination that is max 10 / 0.1 mLor 100 colonies / 1 mL.

Identification of *Escherichia coli* bacteria

Tests in biochemical tests include various tests to determine the activity of microbial metabolism. Observation of metabolic activity is known to be the ability of microbes to use and decompose complex molecules such as starches, fats, proteins, and nucleic acids.

Table 1 Biochemical Test Results

Assays	Sample A	Sample B	Sample X
Indol	+	-	+
Methyl Red	+	-	+
Voges Prekauer	-	-	-
Citrat	-	-	-

Indol Test

The Indole test aims to identify the ability of bacteria to produce indole by using the enzyme tryptophanase. Indole production in the media is possible because of tryptophan. Tryptophan is an essential amino acid, which is oxidized by several bacteria which results in the formation of indole, pyruvic acid, and ammonia.

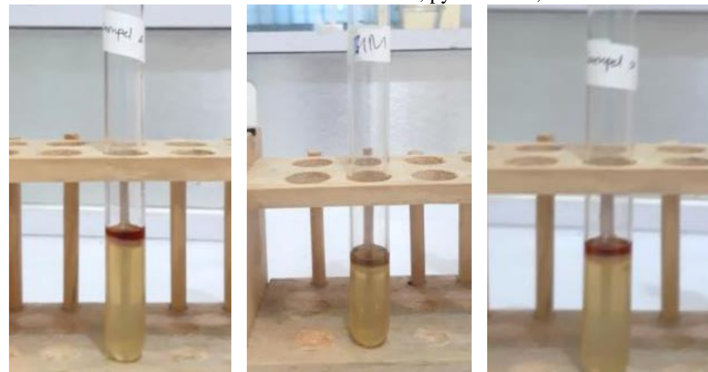


Figure 2 Indol Test sample A (+) **Figure 3** Indol Test sample B (-) **Figure 4** Indol Test sample X (+)

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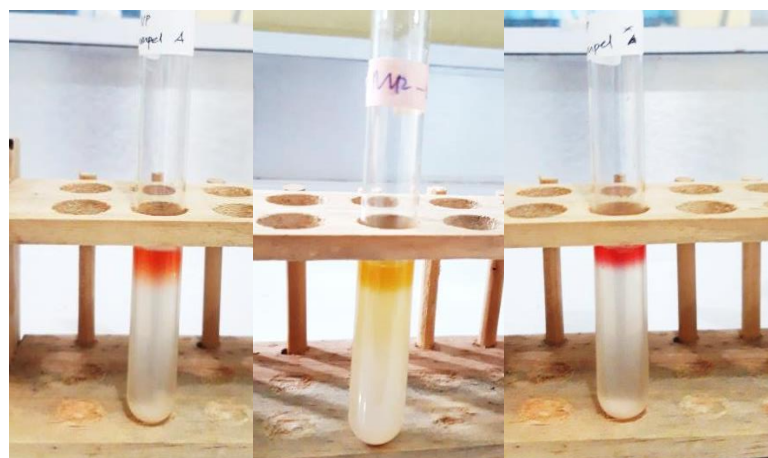


Figure 5 The Methyl Red Test sample A (+) **Figure 6** The Methyl Red Test sample B (-) **Figure 7** The Methyl Red Test sample X (+)

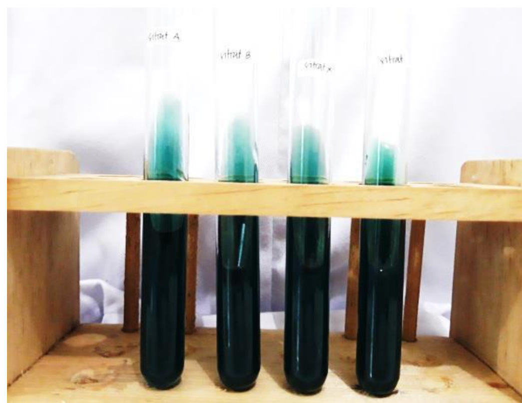
The Voges Proskauer (VP) test

The Voges Proskauer (VP) test is a test used to detect acetonin in a liquid culture of bacteria. This test is done by adding alpha-naphthol and potassium hydroxide. The red color indicates a positive result, while the yellow-brown or colorless color is a negative result. This test is negative for Escherichia coli because Escherichia coli ferments carbohydrates into acidic products and does not produce neutral products such as acetonin.

Figure 8 The Voges Proskauer test sample A, B dan X (-)

The citrate test

The citrate test aims to detect the ability of an organism to utilize it as the only source of carbon and energy. If the bacteria are able to use citrate as their carbon source, it will increase the pH and change the color of the culture medium from green to blue.



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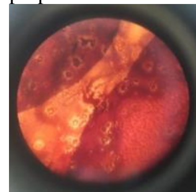
Observation results for the citrate test yielded negative results indicated by the absence of color changes in the citrate test media. Biochemical tests are carried out to determine the physiological properties of bacteria. Based on biochemical tests the results showed that *Escherichia coli* bacteria were found in samples A and X. Positive tests on samples A and X were marked by the formation of a red ring on the upper layer of the indole test and the methyl red test. For negative results, sample B shows the results of non-*Escherichia coli* bacteria or other coliform bacteria, this negative result is also marked by the formation of yellow rings in the upper layer of the indole test and methyl red test. However, the results of this biochemical test have not shown specific results. Therefore the next test is gram staining and observation of the sample under a microscope. UHT milk samples that produce positive results indicate incompatibility with the provisions of the SNI in 2009 and SNI in 2014 that is contrary to the maximum limit of microbial contamination *(**Martin et al., 2011**)*. This can occur because the process of making UHT milk in sample A does not meet the applicable requirements and the storage is not in accordance with the provisions or unfavorable research treatments such as samples contaminated with bacteria from outside during processing **(Boor *et al*., 2017).** The test results on UHT milk samples indicate the presence of Escherichia coli bacteria is not in accordance with a study conducted by **(Cutrim *et al*., 2017**) examining UHT milk against Escherichia coli bacteria which results in no microbial contamination in UHT milk. Whereas UHT milk in sample B produced negative results showing conformity with the conditions specified for the process of making UHT milk. These results indicate that the manufacturing process in sample B follows the applicable terms or conditions **(Coppa *et al.,* 2013)**. So the product produced is good because UHT milk is milk with a manufacturing process using heating techniques at high temperatures allowing the bacteria contained in the milk to die. For yogurt (sample X) that produces positive results containing Escherichia coli bacteria is a sample of fermented milk **(Aryana & Olson, 2017)**. This happens that the possibility of fresh milk used in the manufacture of fermented milk has been contaminated with Escherichia coli bacteria. So the product produced contains *Escherichia coli* bacteria **(Butler *et al*., 2011)**. Because fermented milk is milk that is inoculated with lactic acid bacteria, the results of the methyl red test produce positive results because the lactic acid bacteria itself is able to ferment sugar into lactic acid **(Boor *et al.*, 2017)**. Another cause of the presence of Escherichia coli bacteria in the sample is the possibility of antibacterial activity against undesirable bacteria such as *Escherichia coli* **(Martin *et al*., 2011)**. This can happen because the lactic acid production process runs fast so that the growth of other microbes that are not used in mixing can be inhibited like Escherichia coli. This statement is not necessarily true whether the bacteria contained in the sample used are Escherichia coli bacteria or other bacteria. For this reason, antibacterial activity testing must be done on the sample **(Koushki *et al*., 2016)**.

Gram Staining and Microscope Test

Gram staining is used to identify bacteria. Bacteria stained by the gram method are divided into two groups, namely gram-positive bacteria and gram-negative bacteria. Gram-negative bacteria are bacteria that do not maintain purple dyes in gram staining. While gram-positive bacteria are bacteria that will maintain a dark purple color after rinsing with alcohol **(Tjatur *et al*., 2015).**



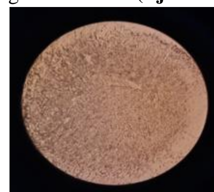
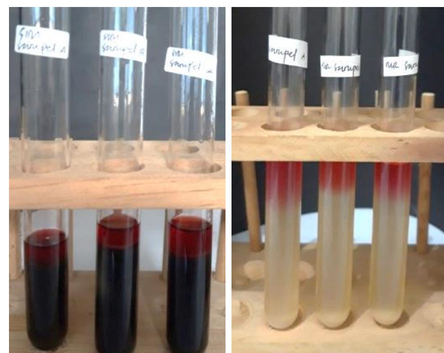


Figure 10 Preparations that are too thick (sample A)

Figure 11 Preparations that are too thick (sample B and X)

The results of gram staining and testing under a microscope showed that the morphology did not appear to be due to the process of making preparations that were too thick (Figure 10), making preparations that were too thin (Figure 9), preparations that were not completely dry, bacteria that did not carry over from bacterial culture at the time of collection using an ose needle and improper treatment. It cannot provide information about the color of bacterial cells or show the nature of gram bacteria and cell shape. Errors in making preparations have also been explained by **(Widyastuti *et al.*, 2014)** that things that often fail microscopy testing is excessive culture taking, preparations that are too thick, preparations that are too thin, preparations that have not been completely dry resulting in microorganisms which were observed to be poorly formed, as well as a less aseptic treatment aimed at avoiding contamination. In these results did not show specific results, then conducted a comparison test with samples inoculated with *Escherichia coli* bacteria **(Butler *et al*., 2011)**.



Figfure 12.a Figure 12.b

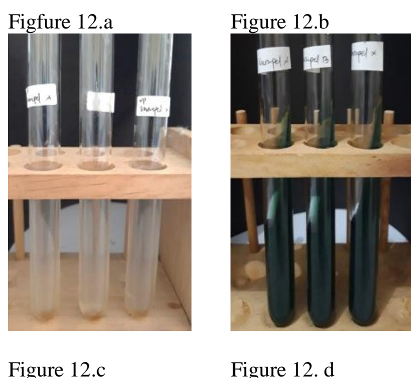


Figure 12.c Figure 12. d

Figure 12 Observation of comparative test results (a) indole (+), (b) methyl-red (+), (c) voges prokauer (-), (d) citrate (-)

In the picture above can be seen the results of the four tests obtained two tests showed positive results and two tests showed negative results. This is by the applicable provisions that the test that shows the presence of *Escherichia coli* bacteria is by obtaining a positive result in the indole test and methyl red test, and the next two tests show negative results.

CONCLUSION

1. Based on the research that has been done, it can be concluded that the sample containing *Escherichia coli* bacteria is contained in sample a uht milk and sample X which is yogurt / fermented milk.
2. the results of the calculation of the colony by the SNI of 2009 concerning the maximum limits of microbial contamination in food namely 10 colonies / 0.1 mL or 100 colonies /1 mL.

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INCIDENCE OF SENSITIVITY OF HELICOBACTER PYLORI IN RAW AND POWDERED MILK TO SOME ANTIBIOTICS GROUPS USED IN EGYPT

Yasser S. Wafy

Address (es):
Fellow, Assiut University Hospitals Egypt.

*Corresponding author: Yasserwafy2014@yahoo.com

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ABSTRACT

A total of 120 random samples of raw milk were collected from dairy farms, farmers houses, street vendors and milk powder (30 samples each) in clean, dry and sterile containers from different localities in Assiut city, Egypt. From January to October, 2018. The results showed that the Incidence of *Helicobacter* species in the examined raw milk collected from (dairy farms, farmers houses, street vendors) and milk powder samples were 4 (1.33 %) , 3 (1.00%) , 5 (1.66 %) and 1 (0.33 %) respectively , While the Incidence of *Helicobacter pylori* in the same samples of examined raw milk and milk powder samples were 2 (0.66 %) , 2 (0.66%) , 3 (1.00 %) and 0 (0.00 %) respectively , That means that the frequency distribution of *Helicobacter pylori* to *Helicobacter* species *i*n the examined raw milk and milk powder samples were 50, 66.6, 60 and 0.00 % respectively , and the Degree of sensitivity of *Helicobacter pylori* to both Tetracycline 30mg , Cephalothine 30 mg and Ampicillin 10 µg were high sensitive (100 %) , while that the Degree of sensitivity of it to Clarithromycin 15 mg and Erythromycin 5 µg were moderate sensitive (75 %) and low sensitive to Augmentin 10 mg (50 %) . in other hand *Helicobacter pylori* were Resistant to both Amoxicillin 10 mg and Unasyn 20 mg .The healthy importance of *Helicobacter pylori* and methods of control are discussed.

Keywords: *Eschericia coli*, UHT Milk, Fermented Milk, IMVIC, TPC, yogurt

INTRODUCTION

Diarrhea can occur due to food and beverage poisoning, with the highest cause being the result of infection with various bacteria, viruses, or parasites. Bacteria that can cause this disease are Escherichia coli bacteria which are known as good bacteria in the digestive tract. Bacteria *Escherichia coli* is a species of bacteria with natural habitats in the digestive tract of humans and animals. But in reality in microbiology, not all types of Escherichia coli are good bacteria **(Delmas, 2015**). Milk is a special food because of its delicacy and balanced composition. Milk contains substances needed by the body such as protein, fat, carbohydrates, minerals, and vitamins. UHT milk is one type of milk that has been processed. While fermented milk is milk with the addition of good bacteria needed by the body in the digestive system **(Delmas, 2011)**. The pollution that occurs in milk is caused by cows, unclean tools and unclean storage areas, dust, air, flies, and poor handling of personnel. After being released from cows, the content of microorganisms in milk is a function of age that determines the level of development of natural flora, the handling of milk that determines the types of organisms carried, and the storage temperature that determines the breeding speed of all types of organisms **(Dumalisile *et al.*, 2005).** High nutritional substances found in milk are good media for microbial growth. Microbial growth causes milk can not stand stored for long and is easily damaged. One way to preserve milk is fermentation treatment (Delmas, 2016). Fresh liquid milk which is widely used is UHT (Ultra High Temperature) milk. UHT milk is a milk product that is obtained by sterilizing milk at a minimum temperature of 135°C for 2 seconds, without the addition of permitted food ingredients and aseptically packaged. This type of milk is usually packaged in a cup or glass with a variety of flavors. UHT milk is also packaged using cardboard boxes or shaped pads ***(Fischer et a*l., 2011**). UHT milk and fermented milk are easily found by the public, both in small shops and large stores because these products are sold in the form of ready to drink packaging **(Widodo *et al*., 2017**). As a liquid beverage packaging product, UHT milk consumption tends to be more desirable than pasteurized milk. While fermented milk is also a product that is in demand because of its usefulness to the digestive system. So for the selection of pasteurized milk products tend to be less desirable because of constraints on the distribution channel **(Omara et al., 2018).** Pasteurized milk which requires the presence of a cold chain (cooling lane), tends to be durable and easily damaged. Bacterial pollution that occurs in liquid milk packaging that is after the packaging is opened. This can cause digestive disorders due to the influence of microorganisms that grow in the milk and some infection conditions such as urinary tract infections in children and infections of the digestive tract **(Dumalisile *et al*., 2005).**

MATERIAL AND METHODS

Materials

The materials used in this study were UHT milk (samples A and B) and fermented milk (sample X) obtained from supermarkets in the Karawang area. Bacterial growth media are NA (Nutrient Agar), SIM (Sulfide-Indole-Motility), and MR-VP (Methyl Red-Voges Proskauer). The reagents used are Kovac reagents, methyl red reagents. Gram staining is the crystal violet, 96% alcohol, safranin, and aquadest.

Methods

Total Plate Count (TPC)

At Each Sample 1 mL is taken, then diluted using distilled water until dilution to 10^{-6}. Furthermore, From Each Sample 10^{-6} Dilution, 1 mL is taken to be inserted into a sterile petri dish, then poured nutrient agar (NA) liquid media and homogenized by sliding horizontal plates or forming the number eight and allowed to freeze. Incubation on milk 37ºC for 24 hours and all colonies that grow are counted as TPC.

Biochemical Test

From the dilution tube, make a scratch on the nutrient agar (na) media, and incubate at 37ºC for 18-24 hours. Observe the murky white colonies of NA.

Indol Test

Colonies from na media were inoculated in a sim-filled tube and incubated at 35ºC for 24 hours ± 2 hours. Then add a few drops of the kovac reagent until a red ring appears on the top layer for positive results and yellow rings for negative results.

Methyl Red (MR) Test

Colonies from na media were inoculated in tubes containing 10 ml mr-vp and inoculated at 35ºc for 48 hours ± 2 hours. Then add 2-5 drops of the mr indicator to the tube. Observe the presence of red for positive results and yellow for negative results.

Voges Prokauer Test (VP)

Pure bacterial isolate inoculated on mr-vp media and incubated for 24 hours at room temperature. Add 0.6 ml of 5% alpha naphtol solution followed by 0.2 mL of 40% KOH. Observe the color change for 30 minutes.

Citrate Test

Inoculation of pure bacterial isolate by zig-zag scratch using ose or using inoculation needle on simmons citrate media to tilt, then incubate for 24 hours at room temperature. Observe the color change from green to blue.

Gram Staining

In NA, take a murky white colony with ose. Then place it on the glass preparation, fix it on the fire bypassing the glass preparation on the fire twice. Drop gentian violet until the entire circle is covered, wait for 5 minutes. Clean over running water. Then drop the lugol and wait for 1 minute. Clean again above running water. Drop alcohol on the entire surface until no color wears off again. Clean again above running water. Drop safranin and wait for 2 minutes.
Clean again above running water. Dry preparations on a tissue.

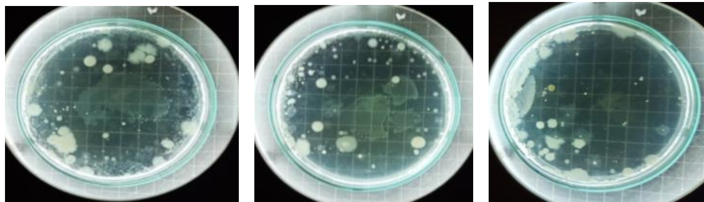
Microscopic examination

The first drop of immersion oil. Then check the preparations under the microscope of the smallest magnification first. After finding the colony lay, change the magnification up to 100 times. The appropriate form of escherichia coli is red, short trunk shape, and a single colony.

RESULTS AND DISCUSSION

Number of UHT Milk and Fermented Milk Colonies

Testing the number of colonies is done by using the cup calculation method with a 10-6 dilution. In the calculation of the number of colonies test results obtained are shown in Figure 1 with calculations using the cup calculation method.



Sample A	Sample B	Sample X
45 colonies	60 colonies	38 colonies

Figure 1 Calculation of the number of colonies in dilutions 10^{-6}

From the data above sample X which is a sample of fermented milk has the lowest number of colonies compared to samples A and B which are UHT milk samples. This can happen because the level of dilution is carried out low so that the growth of microorganisms becomes very numerous and difficult to do calculations, whereas for samples A and B the level of dilution is carried out high **(Caplan & Barbano, 2013)**. For samples A and B which are flavorful and non-flavored UHT milk, the results of the calculation of the number of colonies are in accordance with SNI provisions in 2009 concerning the maximum limit of microbial contamination, namely for flavored and non-flavored UHT milk <10 colonies / 0.1 mL or 100 colonies / 1 mL, while for sample X which is fermented milk is also in accordance with the provisions of SNI in 2009 concerning the maximum limit of microbial contamination that is max 10 / 0.1 mLor 100 colonies / 1 mL.

Identification of *Escherichia coli* bacteria

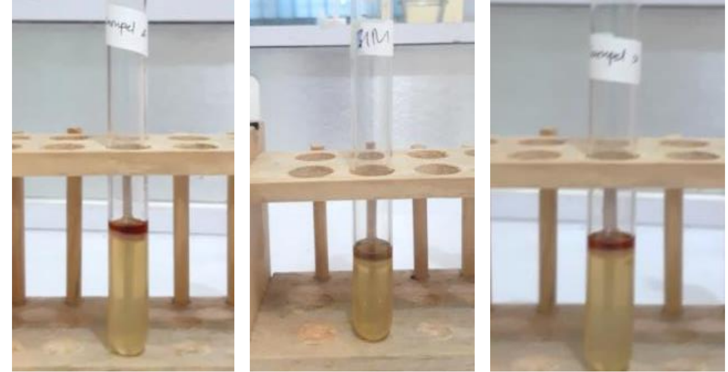
Tests in biochemical tests include various tests to determine the activity of microbial metabolism. Observation of metabolic activity is known to be the ability of microbes to use and decompose complex molecules such as starches, fats, proteins, and nucleic acids.

Table 1 Biochemical Test Results

Assays	Sample A	Sample B	Sample X
Indol	+	-	+
Methyl Red	+	-	+
Voges Prekauer	-	-	-
Citrat	-	-	-

Indol Test

The Indole test aims to identify the ability of bacteria to produce indole by using the enzyme tryptophanase. Indole production in the media is possible because of tryptophan. Tryptophan is an essential amino acid, which is oxidized by several bacteria which results in the formation of indole, pyruvic acid, and ammonia.



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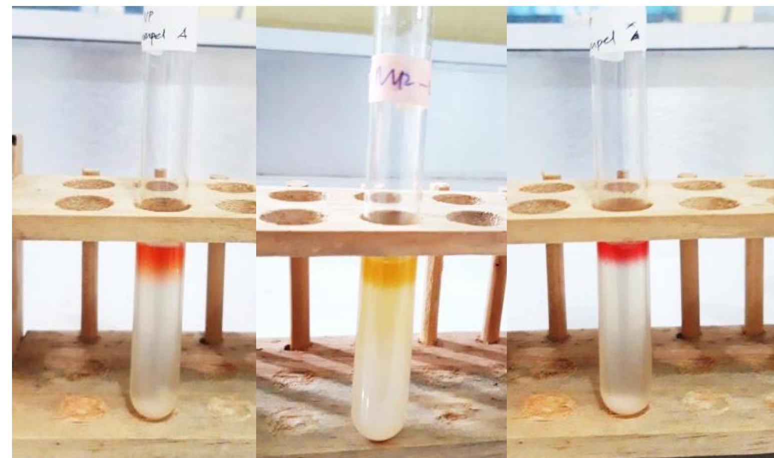

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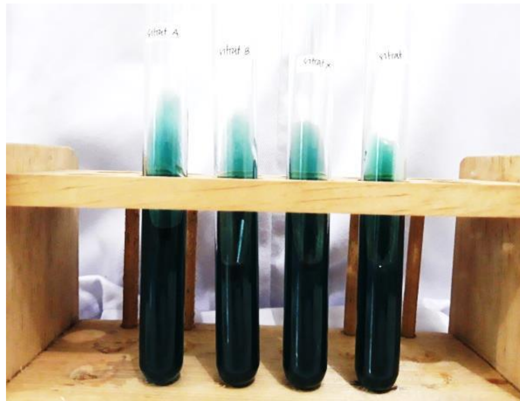
Figure 8 The Voges Proskauer test sample A, B dan X (-)

The citrate test

The citrate test aims to detect the ability of an organism to utilize it as the only source of carbon and energy. If the bacteria are able to use citrate as their carbon source, it will increase the pH and change the color of the culture medium from green to blue.



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Gram Staining and Microscope Test

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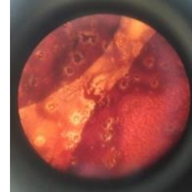
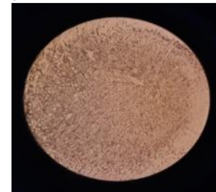




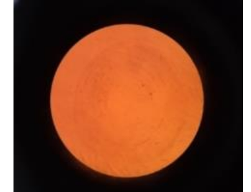
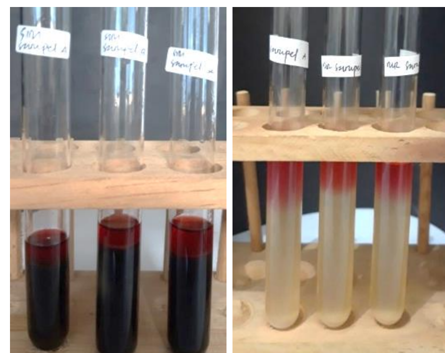


Figure 10 Preparations that are too thick (sample A)

Figure 11 Preparations that are too thick (sample B and X)

The results of gram staining and testing under a microscope showed that the morphology did not appear to be due to the process of making preparations that were too thick (Figure 10), making preparations that were too thin (Figure 9), preparations that were not completely dry, bacteria that did not carry over from bacterial culture at the time of collection using an ose needle and improper treatment. It cannot provide information about the color of bacterial cells or show the nature of gram bacteria and cell shape. Errors in making preparations have also been explained by (**Widyastuti *et al.*, 2014**) that things that often fail microscopy testing is excessive culture taking, preparations that are too thick, preparations that are too thin, preparations that have not been completely dry resulting in microorganisms which were observed to be poorly formed, as well as a less aseptic treatment aimed at avoiding contamination. In these results did not show specific results, then conducted a comparison test with samples inoculated with *Escherichia coli* bacteria (**Butler *et al*., 2011**).



Figfure 12.a Figure 12.b

Figure 12.c Figure 12. d

Figure 12 Observation of comparative test results (a) indole (+), (b) methyl-red (+), (c) voges prokauer (-), (d) citrate (-)

In the picture above can be seen the results of the four tests obtained two tests showed positive results and two tests showed negative results. This is by the applicable provisions that the test that shows the presence of *Escherichia coli* bacteria is by obtaining a positive result in the indole test and methyl red test, and the next two tests show negative results.

CONCLUSION

1. Based on the research that has been done, it can be concluded that the sample containing *Escherichia coli* bacteria is contained in sample a uht milk and sample X which is yogurt / fermented milk.
2. The results of the calculation of the colony by the SNI of 2009 concerning the maximum limits of microbial contamination in food namely 10 colonies / 0.1 mL or 100 colonies /1 mL.

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