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INVESTIGATION OF PRESENCE OF MICROFLORA IN DAIRY PRODUCTS:

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India is the highest milk producer and ranks first position in the world contributing 25% of global milk production. The milk production of India has registered 58% increase during the last nine years i.e., during the year 2014-15 and 2022-23 and increased to 230.58 Mn Tonnes in the year 2022-23.

In India from centuries Milk is also considered as a complete food for all age of humans and in Indian sub-continent its products are liked with more preferences over other foods.

In small scale dairy farms due to poor commercialization of dairy sector and improper handling practices, the prevalent unhygienic conditions favours the entrance of many un-desirable and infectious microorganisms including E.coli, S. aureus and many others which affects the overall nutritional and market value of the dairy products. Consumption of such spoiled and infected product or infectious agent or their toxins caused severe intoxications in humans (Murinda et al., 2004; Oliver et al., 2005). Milk is a complex biological fluid and by its nature, a good growth medium for many microorganisms. Microbial contamination can generally occur from three main, sources viz. within the udder, exterior of the udder and the surface of milk handling and storage equipment. The presence of pathogenic bacteria in milk often emerge as a major public health concern, especially for those individuals who still drink raw milk. In this context, bacteria especially E.coli comes in priority due to simple physiological and nutritional requirements and with easy generation time. Although, majority of obtained E. coli strains from milk products are non-virulent, but highly pathogenic strains were also reported in past from many milk born outbreaks that have lethal effects on host. As evident, their intoxication may cause severe intestinal and other disorders in humans (Kaper et al., 2004).

Initial bacterial contamination of milk may occur from milking animal itself, through shedding of microorganisms colonize on its teats canal or an infected udder (clinical and subclinical mastitis) or it gets contaminated later on at various stages from the animal skin, handling persons, equipments used, through extraneous dirt or use of unclean water (Banwart 1989; Hayes et al., 2001), therefore the microbial content of milk is a major feature in determining its quality.

Staphylococcus aureus is one of the major foodborne pathogen causing outbreaks of food poisoning worldwide (Meyrand et al., 1998). Furthermore, S. aureus represents a main cause of mastitis in dairy cattle (Virgin et al., 2009; Lee et al., 2014; Xue et al., 2014). Some of Staphylococcus species are highly heat-stable with their enterotoxins and as suggested there toxicity increases more in foodstuffs due to shielding effect provided (Bergdoll 1983). S. aureus infections shows a large range symptoms such as minor skin defects, formation of pimples, boils, cellulites, toxic-shock syndrome, impetigo, and abscesses to life threatening disease such as vomiting, abdominal cramps, pneumonia, meningitis, endocarditis, and septicaemia (Balaban and Rasooly 2000, Soomro et al., 2003).

Due to the presence of prevalent warm and humid conditions for bacterial favourable growth (Bhatia and Zahoor, 2007), the infection rate is comparatively higher in India causing high economic loss.

In our study the primary objective was to investigate the occurrence of the pathogen i.e. E. coli and S. aureus in milk samples, as beside causing various diseases, it also spread antimicrobial resistance in humans and animals due to its high daily intake, than other food products thus in second phase we determined the antibiotic susceptibility pattern of both the screened out isolates.

Several virulence factors have been reported to be correlated with the symptoms and severity of infections caused by S. aureus and representing major concerns for the foodprocessing industry (Xing et al., 2016). These factors include hemolysins (alpha, beta, gamma, and delta), leukocidin, toxic shock syndrome toxin-1, and staphylococcal enterotoxins (SEs) (Dinges et al., 2000). Staphylococcal enterotoxins are a group of single-chain, low molecular mass proteins that are produced during all phases of growth (Naffa et al., 2006) and are responsible for gastrointestinal symptoms such as nausea, emesis, abdominal cramps, and diarrhea (McLauchlin et al., 2000; Le Loir et al., 2003). Many types of S. aureus enterotoxins have been reported but the major five serological types are SEA, SEB, SEC, SED, and SEE (Omoe et al., 2003; Orwin et al., 2003). SEs are resistant to inactivation by gastrointestinal proteases as well as to heat and therefore may retain their biological and immunological activities even following pasteurization, food processing, and exposure to gastrointestinal proteases (Asao et al., 2003). Therefore, the detection of these SEs is proposed to be a reliable method for the confirmation of staphylococcal outbreaks and determination of the enterotoxigenicity of strains (da Cunha et al., 2007). The enterotoxigenic S. aureus had been implicated in causing contamination of raw milk (Heidinger et al., 2009; Fusco et al., 2011), cheeses (Ertas et al., 2010; Rosengren et al., 2010), ice cream (Gu¨cu¨kog˘lu et al., 2013), and yogurt. The present study was planned to gain better insight into the prevalence of MRSA in raw milk, Damietta cheese, Kareish cheese, ice cream, and yogurt marketed in different locations in Mansoura City, Egypt and to characterize the strains recovered using molecular analysis of marker genes (nuc, coa, and mecA), virulence genes (hla, sea, seb, sec, and tst), and antimicrobial resistance patterns.

S. aureus is a ubiquitous pathogen associated with both human and animal diseases including mastitis, toxic shock syndrome (TSS) and staphylococcal food-poisoning (SFP) (Le Loir et al., 2003). SFP symptoms include sudden onset of nausea, vomiting, abdominal cramps and diarrhoea, and is caused by ingestion of food containing heat-stable staphylococcal enterotoxins (SETs) (Balaban and Rasooly, 2000). In addition to the classical SET types A to E (SEA–SEE), several new types of SETs or staphylococcal-like (SEls) (Lina et al., 2004), have been described and include SEG–SEI, SElJ–SElQ, SER–SET, and SElU–SElV (Ono et al., 2008). An additional virulence factor is the toxic shock syndrome toxin 1 (TSST-1) which affects the immune response of a colonized host (Schlievert et al., 1993). In addition, resistant and multi-drug-resistant staphylococcal isolates, e.g. methicillin-resistant S. aureus (MRSA) have been isolated from both humans and animals (Morgan, 2008; Miller and Kaplan, 2009). A risk assessment indicated that entero toxigenic S. aureus in fresh and short-time ripened raw milk cheeses may pose a health risk since a large fraction of cheeses could contain unsatisfactory levels of S. aureus at the time of consumption (Lindqvist et al., 2002). However, several limiting data gaps were identified, e.g. occurrence and levels of S. aureus and enterotoxins in cheese, sources of contamination, and pathogenic potential of S. aureus strains.

To counter these living threat agents, several measures especially administration of antimicrobials are employed globally. Antibiotics are natural, synthetic, or semisynthetic substances that interfere with the growth or killing of microorganisms, specifically bacteria, and are used to treat or prevent infections in humans and animals. Antibiotics are now an “endangered species” facing extinction due to the worldwide emergence of antimicrobials resistance (AMR) and the void in the development of new therapeutic substances [J. O’Neill, 2019 , WHO 2015]. Nowadays, a global analysis of antimicrobial usage revealed that the worldwide consumption of antimicrobials in food animal production is estimated at ≥57,000 t (1,000 kg) and projected a 67% increase in total usage by 2030 to ≥95,000 t [9].

Shiga-toxin producing E. coli (STEC) and Enterotoxigenic E. coli (ETEC) were associated with several life-threatening food-borne outbreaks worldwide [Blanco J at.el., 1993, Majowicz S at.el., 2014]. STEC produces cytotoxins encoded by stx1 and stx2 genes. These cytotoxins are associated with serious human illnesses as hemorrhagic colitis (HC) and hemolytic uremic syndrome (HUS) that usually end up with fatal consequences. STEC causes around 3 million cases of acute illness and over 200 deaths each year. ETEC causes diarrhea via production of heat-stable and heat-labile enterotoxins encoded by ST and LT genes, respectively. ETEC isolates are the most common cause of endemic diarrhea in children in developing countries (El-Gendy A at.el.,2013]. In addition, it is the most frequent etiology of travelers' diarrhea [8].

Nowadays, molecular typing methods are crucial in epidemiological investigations of food processing and enhance the resolution of surveillance (Wu et al., 2015). Of various subtyping approaches, multilocus sequence typing (MLST) is a widely accepted method of DNA sequence based typing that based on analysis of relatively conserved genes that encode essential proteins. For S. aureus, the level of discrimination provided by MLST is sufficient to provide a relatively detailed picture of the global dissemination of the organism (Saunders and Holmes, 2007).

Material and Methods:

Collection of Sample:

The microbial analysis was conducted using two dairy products 1. Paneer 2. Yogurt

A two sample of dairy products were collected in sterile polythene. The sample were collected from daily needs dairy of Nagpur.

Experimental material and sterilization:

Different laboratory material like petridishes, test tube, conical flask, beaker etc. Being used for the experimentation were washed with detergent, rinsed with clean water and then dried. This was followed with a proper sterilization of the material in hot air oven and autoclave.

Media Preparation:

The media used for the isolation and characterization are Nutrients agar, Baired Parker agar, Maconkey agar, Citrate agar, Methyl red, voges prosker respectively. Isolation and Inoculation and Incubation: The media such as nutrients agar, Maconkey agar, Baired Parker agar, are poured Asceptically into the petridish and allow to solidify. After solidification, the sample is taken with swab stick and inoculated asceptically on the petriplates. This petriplates are allow to incubate aerobically at 37°c for 24 -48 hrs.

Microscopy and colonial identification:

Characterization and identification of the colony isolates was achieved by al morphological examination of the colonies in the plate (microscopy) for colonial appearance, size, elevation, form, edge, consistency, color, opacity, hemolysis and pigmentation hence results were recorded. The isolates were identified and characterized based on their cultural characteristics, gram reaction and biochemical reaction as follows.

Gram Staining:

This is the most important widely used procedure for characterizing organisms. It was first described by Christian Gram. This method divides the organisms into two groups, Gram positive which is purple in color and Gram negative which is pink in color. This technique is based on the ability of organisms retain primary stain (crystal violet dye) during decolourisation with alcohol or actone. Gram positive organisms retain primary stain while Gram negative organisms are decolourised by alcohol and takes up the red colour counter 19 stain. A smear of an isolate was made on clean slide and allowed to dry. It was then heat fixed by passing the smear though the bursen burner, this is done to enhance the sticking of the organism on the microscope slide. The smear was flooded with crystal violet and left for 60sec before washing off with water. Lugols iodine was added and allowed to stand for 60 sec before being washed off and decolourised with alcohol for 10 seconds. The slide was then washed off, stained with safranin for 30 sec washed off and allowed to air dry. A drop of immersion oil was added to the slide which was then viewed under the microscope using the x 100 objective lens.

Characterization of bacteria (Biochemical Tests)

Motility Test:

Motility test is aimed at identifying motile bacteria. Motility can sometimes be referred to as the way an organism grow on solid media and it determine the presence or absence of flagella. Each bacteria isolated was separately inoculated into a semi solid medium using sterile straight wire and incubated at 37°C for 24hrs. Migration of the ates away from the line of inoculation was a positive result while lack of migration away from the line of inoculation indicated a negative result.

Catalase Test:

This was used to differentiate those bacteria that produce enzyme Catalase. A little portion of the bacterial growth was transferred with a sterilized wire loop to a drop of hydrogen peroxide on a clean glass slide. The presence of catalyses observed by bubbling indicated a positive test result while absence of bubble indicated negative est result ofbacteria.

Indole Test:

This test was carried out for Indole production by test organism which is important in identifying entero bacteria. The test was carried out as described by a e portion of each isolate was inoculated onto 5ml of sterile peptone-water enriched with 1% tryptophan and incubated at 37°C for 24 hours. To the culture, 0.5ml of Lovac's reagent was added and gently stirred. A red color indicated positive result while a yellow color indicated negative test result of bacteria.

Methyl Red test (MR):

MR test was carried out to identify Enterobacteria based on the ability is produce all maintain stable end product from glucose fermentation. It was carried out is described by (Ochei and Kolhatkar, 2000). A little portion of each isolated into the glucose phosphate peptone water medium and was incubate at 37°C for 48 hours. Few drops of methyl red were added to the culture MR positive indicated by red color. Vogus proskaeur Test The test organism was introduced into glucose phosphate. Peptone water was incubated at 37°C at 48hr. 5 drops of Barrites A (alpha napthol) and Barries B (potasssium hydroxide) reagent were added, mixed and the result read. A pink coloriation indicated a positive result.

Citrate utilization test:

This test is based on the ability of an organism to use citrate as it is source of carbon A little portion of each isolate was inoculated into Koser's citrate medium and incubated at 37°C for 72 hours. A positive citrate test was confirmed by formation of bright blue color while the initial green color of the medium indicates a negative test.

Coagulase test :

A portion of an isolate was picked using a sterile wired loop and emulsified in physiological saline solution on a clean grease- free slide to give a thick suspension and mixed well. The formation of macroscopic clumps within 10-15 seconds indicated a positive result while the absence of macroscopic clumps indicated a negative result. Fermentation test: This determines

the ability of organisms to ferment sugars including glucose, mannitol, 21 sucrose, etc. phenol red broth was prepared and sugars were incorporated at a final concentration of 1%. To detect gas production, Durham tubes were placed in inverted positions in each test tube. Each test isolated was inoculated and incubated at 370c for 24 hours. An un-inoculated medium was used as control. Utilization of the sugars was indicated by formation of a yellow Coloration on the medium while production of gas was shown by creation of space at the end of the Durham tubes. Organisms used for the fermentation test were grown in a medium having the following composition: Peptone 10% Nacl 0.1% Bromocrosol sugar 1.0% Fermentation sugar 1.0%.

Antibacterial Sensitivity Testing:

The antibacterial sensitivity test was used to determine the susceptibility of various bacterial species to various antibiotics and synthetic agents. A standardized disk diffusion method was used; antibiotic susceptibility testing and the zone size interpretation chart were used for the determination of the bacterial sensitive to the antibiotics selected. The commercially prepared paper disk was impregnated with the various antibiotics that will be assessed against the isolates; gentamycin (Gen), chloramphenicol (C), ampicilin (Amp"), penicillin-G (P^1), tobramycin (Tob) and cefpodoxime (CPD). The plates were incubated at 37'e for 24hours after which zones of inhibition was measured and interpreted. Results obtained were classified as resistant or sensitive.

Results:

On inoculating the sample of dairy products on different media following result are obtained.

Table : Characteristics of microorganism on different media

Organisms	Media	Color	Margin	Elevation	Density	Shape
KD1	Nutrients agar	Pale yellow	Smooth	Convex	Opeque	Circular
	Macconkey agar	Pink	Entire	Flat	Opeque	Circular
	Baired parker	Blank	Entire	-	Opeque	Circular
KD2	Nutrients agar	Pale yellow	Smooth	Raised	Opeque	Irregular
	Macconkey agar	Pink	Entire	Convex	Transparent	Circular
	Baired parker	Black	Entire	-		Circular

Table 4.2 biochemical test for bacteria isolates and identified.

Organisms	Gram reaction	Catalase	Motility	MR	VP	Indole	Citrate	Coagulase
KD1	+ve	+ve	non-Motile	+ve	+ve	-ve	+ve	+ve
KD2	-ve	+ve	Motile	+ve	-ve	+ve	-ve	-ve

Description:

The sample KD1 from its biochemical and morphological character it is *staphylococcus aureus* which is gram positive, cocci, non motile and coagulase positive.

The sample KD2 Is *e.coli* a gram -ve rod shaped coliform bacteria which is motile andcatalase positive.

On Inoculating the sample of dairy products on different media following result obtained.

Fig.4.1 Nutrient Agar

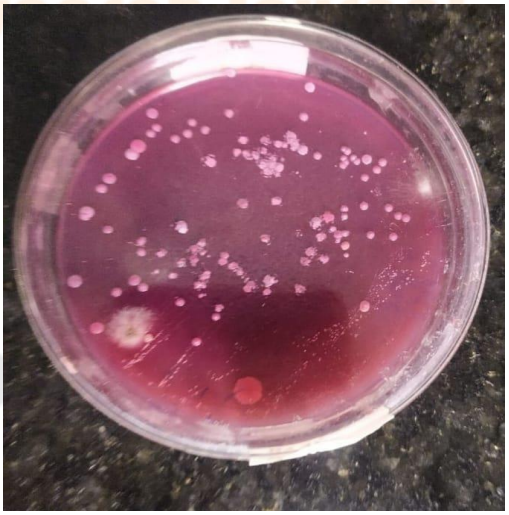

Fig.4.2 Macconkey agar

Fig 4.3: Baired parker

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Table 4.3 Number And types of isolated from sample analyzed

Isolates			
Sample	***S.aureus***	***E.coli***	**Total**
Panir	2	1	3
Yogurt	3	2	5
Total	5	3	8

Table 4.4 occurrence of isolated and frequency of Isolation

Microbial isolation	Occurrence of isolated	Frequency of obtained
S.aureus	5	62%
E.coli	3	37%

The frequency of the occarance relevant that *S. Aureus* has the highest isolated than *E. Coli*.

Table. 4.5 Biochemical characteristics of micro orgranism

Test	S.aureus	E.coli
Motility	Non motile	Motile
Shape	Cocci	Rod
Oxygen requirements	Anaerobic	Anaerobic
Indole	-ve	+ve
MR	+ve	+ve
VP	+ve	-ve
Citrate	+ve	-ve

Fig 4.4. Biochemical test for *S.aureus*.

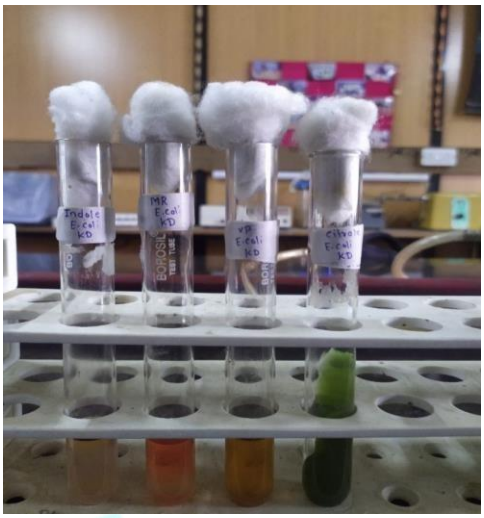

Fig4.5: Biochemical test for *E.coli*

Phenotypic confirmation of microorganism

1. *S aureus*

Gram Staining:

Fig 4.6 *S.aureus*

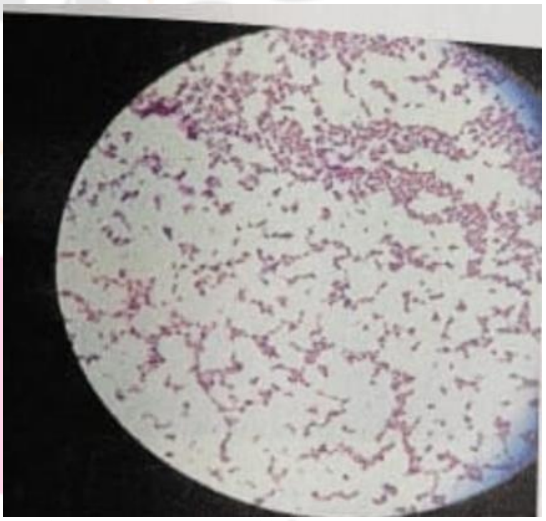

Fig4.7: Gram + cocci

2. *E.coli* **Gram Staining:**

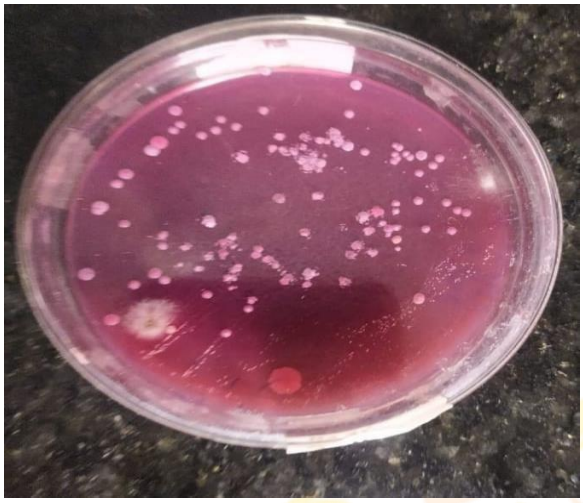

Fig 4.8: *E.coli*

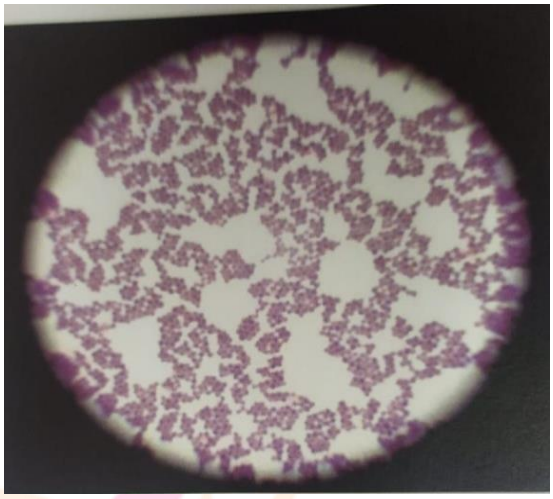

Fig 4.9: Gram -ve cocci

DISCUSSION

The result of the study show that the it was found that all milk products contaminated. This may be due to unhygienic handling of milk. In case of milk products the highest contamination was recorded in Yogurt. The organisms with highest prevalence rate wass.aureus.

The difference in the prevalence rate of micro orgranism in milk and milk products may origin from the method of manufacture, storage and handling. The presence of *e.coli* and s.aureus will render milk unfit for human of these organisms will cause infection and intoxication. *Staphylococcus aureus* food poisoning is a major concern in public health program *s.aureus* present in milk products as a result of milk collected from the animal suffering from disease condition. Hygenic condition during production and post processing should be improved. The professionals should apply and control of the disease. The professionals should in from the public about the relevance of milk pasteurization before consumption to avoid food born infection.

The existence of high conc. of *e.coli* in milk products indicated the relatively poor quality of milk related with standard hygiene of the farm management milk collection and processing system.

CONCLUSION

The result of the present study of milk and milk products were found contaminated with *staphylococcus aureus* and *E.coli* which is cause the may food poisoning and pose a treat to public health. It is indicated a need for more strict hygenic practice, regular sterilization of dairy equipment washing of utensils, milker'hsnds. Udder, eradication, of disease animal, pasteurization/ boiling milk before collecting and distribution for consumption and products making.

According to HACCP (Hazard analysis and critical control points) guidelines to improve the microbiological quality and safety of milk products. In conclusion, the result of the present study provided that micro bial quality and safety of milkproducts was unsatisfied.

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