FOOD MICROBIOLOGY AND SAFETY

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1.0 Introduction:

1.1 Soft cheese:

Cheese is the most commonly used dairy product and it is being consumed by people across the world. A number of delicacies are prepared from it and hence it has a great demand s in the hotel and the restaurants and in other food and snacks manufacturing industries.The statistics graph of figure 1 suggests that the demand for cheese would increase over time because of its nutritional importance and popularity among the consumers (Statista, 2021). It is prepared by fermenting milk with different organisms by different fermentative lactic acid bacteria. Apart from these lactic acid bacteria there are other bacteria as well which grow on cheese and show their pathogenic activity. *Listeria monocytogenes*, *Staphylococcus aureus*, and *Escherichia coli* are some of the organisms that cause serious food-borne infections that can be found in cheese (Choi *et al*., 2016). Moreover, the organisms like *Clostridium perfringens* and *Bacillus cereus* are observed to grow in the cheese, spoil it and create serious illness (Andersson, Ronner and Granum, 1995). This could put the consumers and the users of cheese at risk. Therefore, it needs to be pasteurised properly to remove these pathogenic organisms. Further, to prepare soft cheese different moulds are used, such as *Penicillium camemberti*, *Penicillium roqueforti* and others. Thus unpasteurised cheese may contain the spores of the moulds which could cause serious health concerns (Fröhlich-Wyder, Arias-Roth and Jakob, 2019). Therefore, in order to increase sales product maintenance has to be done properly.

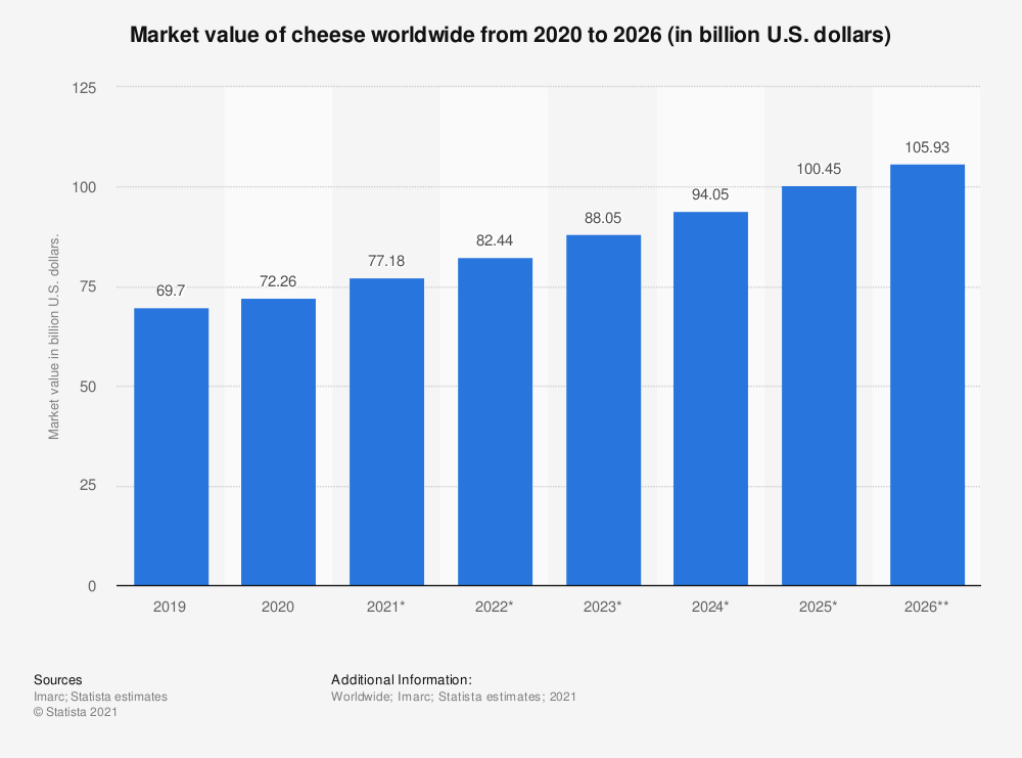

Figure 1: Market Value of Cheese Worldwide from 2020-2026

Source: (Statista, 2021)

1.2 Risk analysis:

Referring to the article of Choi et al. (2016), risk is the probability of getting adverse health issues by using a product and hazard define risk and the severances of the health effect is termed as hazards. The three components of risk analysis include the risk assessment which judges the factors that could increase the risk, risk management by implementing HACCP, GMP and other standards and lastly risk communication which allows the consumers and the suppliers to have a complete knowledge of risks and hazards so that the health consequences can be avoided. Moreover, the components of risk assessment suggest the different factors that are related to the characterisation of the potential risks. The factors such as the hazard identification allows to find out the risks and health threats of the products, hazard characterisation enables in understanding the potential health threats that may cause, exposure assessment analyses the damage that may cause and risk characterisation to determine the levels of risk caused by the product (Haberbeck *et al*. 2018).

1.3 Microorganisms- hazard identifications:

As discussed, there are several organisms involved that cause serious health concerns such as the *Staphylococcus aureus, Listeria monocytogenes,* different Enterobacteriaceae such as *Escherichia coli, Salmonella* sp. The health issues posed by them cause serious hazardous outcomes.

- *Staphylococcus aureus* (gram positive, cocci, facultative anaerobe, found in human skin) has been observed to produce dreadful enterotoxins that cause serious illness with the rapid symptoms of vomiting, diarrhoea and nausea (Kousta *et al*., 2010). In order to remove the organism from milk and milk products proper pasteurisation is required.
- *Listeria monocytogenes*(small non-spore forming gram positive rods, facultative anaerobe, found in soil) is another food borne pathogens that cause serious health concerns such as septicaemia, sepsis, vomiting, nausea, fever or even it may lead to coma among the individuals who immunocompromised or have weak immune system such as pregnant woman and others (Konhorst, 2007).
- *Salmonella* sp such as *Salmonella typhimurium* and *Salmonella enteritis*(gram negative rods, facultative anaerobe, found in poultry farms) have been observed majorly associated with the poultry farms. They can cause salmonellosis with the acute symptoms of diarrhoea, fever, abdominal pain, headache, vomiting and it may also cause chronic issues such as Reiter syndrome, reactive arthritis. Being heat sensitive, it can be killed by pasteurisation (Nasrollahzadeh *et al*. 2017).
- *Escherichia coli (O157:H7)* (gram negative rods, facultative anaerobe, mostly habitat in gastrointestinal tract) is another pathogenic organism that causes enteric diseases with the symptoms of diarrhoea, vomiting and others. Referring to the article of Megawer *et al*. (2021), it suggests *E. coli* can be a threat to the public health due to its causing effect of gastrointestinal diseases. This organism is also suitable to remove by the process of pasteurisation.

1.4 Predictive microbiology:

Predictive microbiology is observed to implement microbiology, statistics, mathematics all together to identify the potential risks and hazards of any food product. It uses different programming languages such as R programming language to identify the health hazards (Liu *et*

al., 2020). It deals with the intrinsic and extrinsic factors of pH, water activity, nutrient content, relative humidity, temperature, presence of microorganisms and others. Therefore, predictive microbiology plays a great role in the food industry by helping in the estimation process of risks and hazards. It helps in understanding the potential risks that could emerge due to the changes in microbial content or any physical and chemical factors and thereby, helps in quality maintenance of the food product (Valdramidis, 2016).

2.0 Materials and methods:

2.1 Product introduction:

Risk analysis of cheese is done which is prepared from goat milk and calf rennet as the key ingredient. The product details like company name, date of purchase and date of expiry is provided in appendix I. The process of producing mould-ripened soft cheese from goat milk follows a series of fermenting steps. The article by Miszczycha *et al*. (2013), suggests the process starts with the collection of goat milk then maturing it for 1 hr at 24°C. The starter culture is added to it and the milk is coagulated for 24 hours using rennet at 24°C. Then Dry Salting is dome on the surface for an hour. Then the product is kept for ripening up to 10 days at 4 °C. Next, dripping is done before packaging at 40°C for the first 10 days, next at 20°C for 2 hours and storage is maintained at 8°C for 35 days. Finally the product is obtained.

2.2 Recovery of microorganisms from cheese

To obtain microorganisms from cheese, the product is first diluted through the process of serial dilution and then the samples are plated using the plating techniques:

Process of serial dilution:Method Booklet (Practical-2 ,Task-2) describes the serial dilution

Media used:

- Nutrient agar to get the overall microbial load
- Violet red bile glucose agar (VRBG) for selectively obtaining Enterobacteriaceae (*Salmonella* and *Escherichia coli*)
- Baird-Parker agar (BP) media for selection of *Staphylococcus aureus*
- De Man, Rogosa and Sharpe agar (MRS agar) for selection of lactic acid bacteria
- Listeria Selective Agar (LSA) for selection of *Listeria monocytogenes*

Procedure for plating: Method Booklet Practical-2 ,Task-3 for plating techniques.

2.3 Identification of isolated microorganisms:

The source of the organism was from our previous plate.

To identify the microorganisms the following steps are followed:

- Streak plate method is used to streak out isolated colonies obtained from the previously cultured plates into other fresh media plates to obtain pure culture of the organisms. In this experiment nutrient agar is used for the streak plate method. This is because the reference from the article of Hallmann, Berg and Schulz (2006), suggests that the nutrient agar contains all sorts of essential nutrients that permits the growth of a range of organisms and they can be differentiated by their colony structure and morphology.
- Transferring the obtained colonies into the agar slopes. This is because in the referred article of Gerloff, Fitzgerald and Skoog (1950), it has been mentioned that culturing in agar slopes or slants helps in obtaining pure culture of the organisms due to less moisture content and less exposure to the outside environment which would help in growth of the microbes without contamination and this method can be useful for long time storage.
- The obtained organisms in the selective media are subjected to gram staining to identify their gram character. According to the referred article of Thairu, Nasir and Usman (2014), the gram staining process is a key and important tool for diagnosing clinical pathogens depending on their gram character and microscopic morphology.

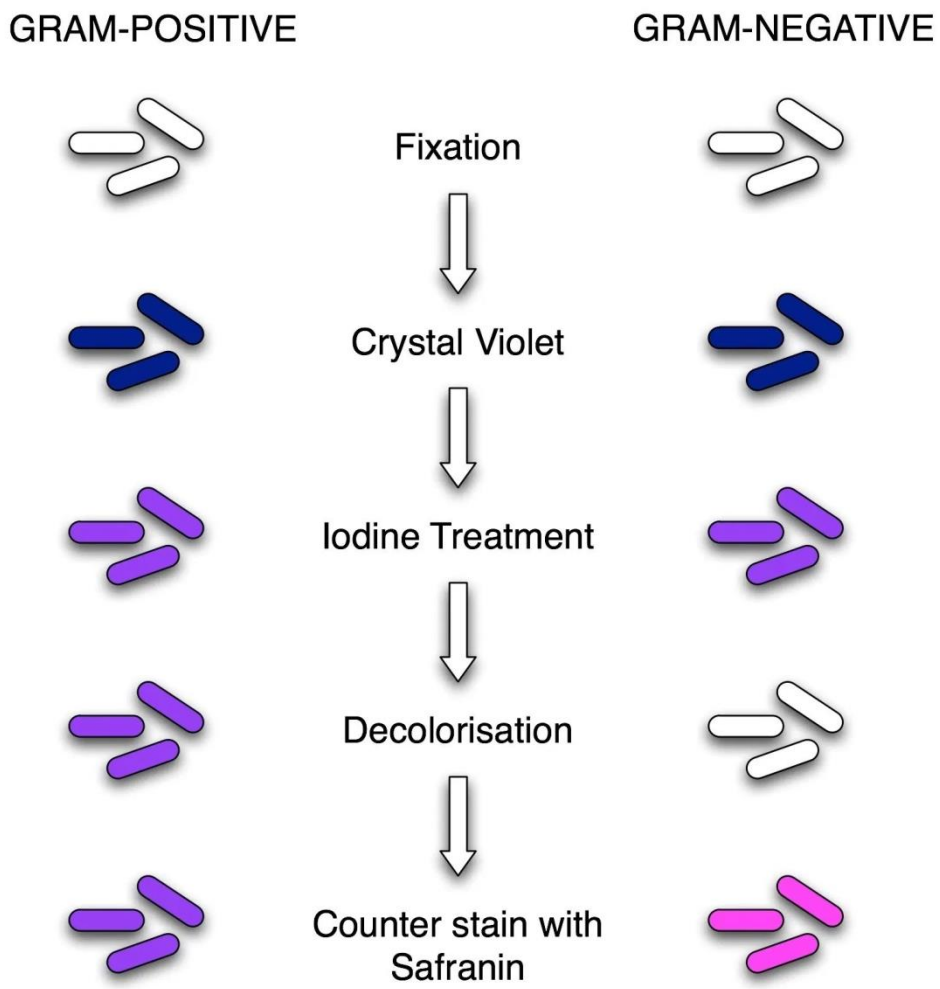

Figure 2: Gram Staining

Source: (Microbe online, 2021)

- Biochemical tests are done for identification of the isolated microorganisms, such as the oxidase test to identify cytochrome oxidase activity which generates a blue colour compound. This helps in identification of particular bacteria as recommended in the referred article of Tarrand and Gröschel (1982).

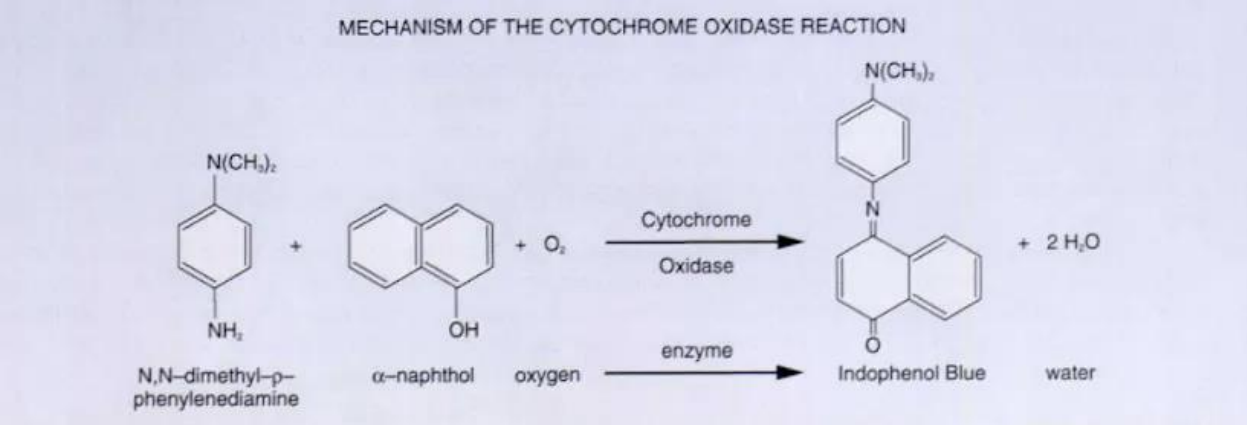

Figure 3: Oxidase test reaction

Source: (Microbe online, 2021)

- Catalase test to check the catalase activity or the presence of catalase enzyme within the microorganisms which is mostly done to identify the enteric organisms. The referred article of Taylor and Achanzar (1972), suggests that the catalase test helps in differentiating and identifying the enteric pathogens in diagnostic procedures.

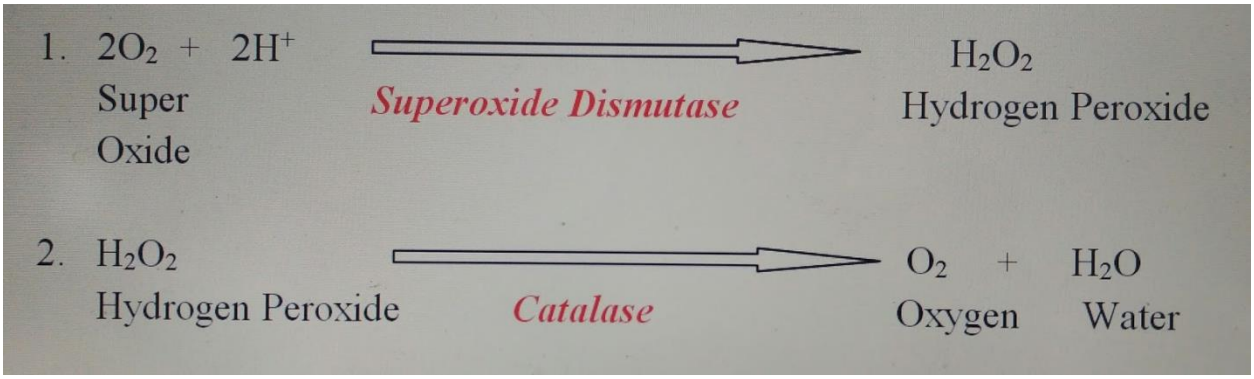

Figure 4: Catalase test reaction

Source: (SIVASAMY, 2021)

- API 20E and API 50 CHL software is used to identify the fermenting ability of the gram negative enterobacteria (Singh and Prakash, 2008)

2.4 Detection of virulence factors (Hazard characterisation)

The organisms cultured in different selective media are taken and then further checked for their ability of virulence. The following experiments are done to check the virulence ability:

Haemolytic activity in Blood agar: There are several microorganisms which have the capability of lyse the blood cells by rupturing its membrane. Depending on the lytic activity of the

microorganisms, three types of haemolysis are observed, namely, alpha, beta and gamma. Microbes such as *E. coli, S. aureus* and others produce beta haemolysis by completely rupturing the blood cells. The colonies of the microbes are streaked over blood agar plates and incubated at 37°C for 24 hours (Buxton, 2005).

Staphylase test or coagulase test for S aureus:

This test determines the coagulation activity of the fibrinogen present in blood cells. Positive coagulation along with catalase activity confirmscoagulase activity of *S aureus.* The test is carried out by dissolving a colony of the organism *S. aureus* in plasma of the blood cells and incubating at 37°C for 24 hours (Fisk, 1940).

Agglutination test for Salmonella:

Latex agglutination test is done for confirming the organism as *Salmonella*. Positive agglutination denotes the organism to be *Salmonella sp*. The organism is mixed with the serum and observed for agglutination within 15 to 20 minutes at room temperature (Barsoum and Awad, 1972).

3.0 Results:

3.1 Numbers of microorganisms recovered (counting dilutions containing 30-300 colonies):

After carrying out serial dilutions in Maximum recovery diluent (MRD) or Ringers diluent, the dilutions are plated in different media like nutrient agar, violet red bile glucose agar (VRBG), Baird-Parker agar (BP) media, De Man, Rogosa and Sharpe agar (MRS agar), Listeria Selective Agar (LSA) and are incubated according to the mentioned time and temperature in appendix III. It has been observed that in violet red bile glucose agar (VRBG) 23 colonies are observed in -1 dilution and 2 colonies in -2 dilution and rest in Baird-Parker agar (BP) media and De Man, Rogosa and Sharpe agar (MRS agar) the colony counts are much high to count which is more than 300 colonies. The result is given in tabulated form in appendix IV.

pH reading of cheese sample:

The pH of the sample cheese is measured at the temperature of 20.4 °C. The unpasteurised cheese has a pH value of 6.15 which is almost neutral and provides a favourable condition for the growth of most of the bacteria, yeasts or moulds.

Table 1: pH and temperature of the cheese sample

Group Number	Name of cheese	Pasteurized / Unpasteurized	pH	Temperature
G-6	Monte Enebro	Unpasteurized	6.15	20.4 °C

(Created by Author)

Water activity (a_w) *reading of cheese sample:*

Water activity of a sample is noted in order to understand the water content of the sample as the more amount of water it would be more favourable for microorganisms to survive

Table 2: Water activity of the cheese sample

Group Number	Name of cheese	Pasteurized / Unpasteurized	a_w	Temperature
G-6	Monte Enebro	Unpasteurized	0.65	22.1°C

(Created by Author)

3.2 Results of Gram stain, oxidase, catalase, API20E, API 50 CHL

Results of Gram staining: The obtained colonies from streak plate method are taken to examine the gram character and morphological characteristics of microorganisms by doing gram staining. According to the article of Thairu, Nasir and Usman (2014), the microbes which appear as purple in colour and gram negative organisms appear pinkish red in colour. Coherent to this, it has been observed that the colonies obtained from MRS agar or De Man, Rogosa and Sharpe agar are gram positive short rods in nature .Colonies from Baird-Parker agar or BP agar show gram positive cocci in bunch which are again present in grape -like clusters. And the colonies from VRBG or Violet Red Bile Agar are observed to be gram negative discrete rods. The results are given in pictorial and tabulated form in appendix V. These results indicate the presence of beneficial and harmful organisms in the cheese sample.

Results of catalase and oxidase tests (Biochemical test): The biochemical tests which are done include the oxidase and catalase test. Among these results are obtained from the catalase test and it has been observed that microorganisms obtained from VRBG or Violet red bile glucose agar (VRBG) are catalase positive indicating presence of *E. coli* or *Salmonella*. Results from the colonies of De Man, Rogosa and Sharpe agar or MRS agar are negative and Baird-Parker agar or BPA produced negative results which strongly indicates presence of *Staphylococcus*. Oxidase test results are negative for all the three (VRBG -ve ,MRS -ve and BPA-ve) indicating the organisms do not possess cytochrome oxidase activity. The images are provided in the appendix VI.

Results of API20E, API 50 CHL:

The results of API20E and API 50 CHL are checked for the presence of enterobacteria which are fermentative or non-fermentative. The results are provided in appendix VII. It is observed from API20E software by sequencing that S*erratia liquefaciens* is the most matched organism while in case of API 50 CHL lactic acid bacteria species called Lactobacillus Plantarum was found for this experiment.

3.3 Detection of virulence factors

Haemolysis test in Blood agar: Appendix VIII indicates the difference in the plate before and after incubation with the microorganisms. It has been observed that after 24hrs of incubation the colour of the media is totally changed and there is clear observation of transparent zones surrounding the bacterial growth depicting beta haemolysis in the plate. Thus, a clear indication of presence of fastidious organisms such as *Staphylococcus aureus* which are highly virulent in nature. This is because the article by Somerville *et al*. (2002), shows that haemolysis is a strong virulence factor among the microbes and *Staphylococcus aureus* possess beta-haemolytic activity.

Staphylase test or Coagulase test: Coagulase tests are positive when the bacteria possess the virulence factor of converting fibrinogen into fibrin present in plasma of the blood which results in the coagulation or clotting which is observed in *Staphylococcus aureus* as per the referred article of Bennett and Monday (2003). The results provided in Appendix IX, suggests that the

coagulase test is positive indicating and confirming the presence of *Staphylococcus aureus* in the sample.

Salmonella test or agglutination test: As depicted in the article of Formal, Baron and Spilman (1954), this test helps in examining the virulent factor of serum agglutination in *Salmonella.*This is an antigen-antibody interaction test which determines the ability to agglutinate in the blood serum. The presence of *Salmonella* organisms indicates a positive test in agglutination. In this experiment, it has been observed that there is a negative result in an agglutination test indicating absence of *Salmonella species* in the sample.

4.0 Discussion:

The experiments are carried out in order to get a specified result of risks associated with the fermentation of yeast. Potential risks are always there in cheese production especially in case of mould ripened cheese. These cheeses are made by using the moulds like *Penicillium* species, which have the potential to produce mycotoxin which can give deadly results in human health. They could be carcinogenic as well. Therefore, the quality testing of the products should be done well (Bennett, 1992). Culturing the sample in De Man, Rogosa and Sharpe agar (MRS agar) for selection of lactic acid bacteria indicates high count of bacteria in the sample indicating the sample that is used is fresh. Further, presence of microorganisms in Baird-Parker agar (BP) media for selection of *Staphylococcus aureus* indicates the presence of the organism in the sample. It could be either because of mishandling or the organism may be present because of being unpasteurized. The pH and water activity of the sample is perfect and as per the standards.

Gram characters depict the presence of different bacteria which are cocci and rod shaped. These could be either beneficial lactic acid bacteria or pathogenic organisms. Moreover, the biochemical tests indicated the presence of catalase activity which again indicates either beneficial lactic acid bacteria or it could be pathogens such as *E coli* or *Staphylococcus.* But the gram character and culture growth indicated absence of *E coli* in the sample. Therefore, further confirmation of *Staphylococcus* is done by performing Blood agar culture and coagulase tests which confirm to be positive. Thus, as per the article of Foster (1996), the organism can be confirmed to be *Staphylococcus aureus* as this organism is gram positive cocci, present in grape like clusters, coagulase positive and causes beta haemolysis, the two lethal virulence factor of the

organism which can lead to death. Therefore, consuming this cheese contaminated with *Staphylococcus aureus* would result in a dreadful outcome of food poisoning.

5.0 Conclusion:

The product that was examined was no doubt fresh but there is presence of *Staphylococcus aureus*, it may have emerged due to handling issues or it could be present because the cheese was not pasteurised. Therefore, Good Manufacturing practices, Good laboratory Practices are mandatory for quality testing and pasteurisation is also required for raw milk products.

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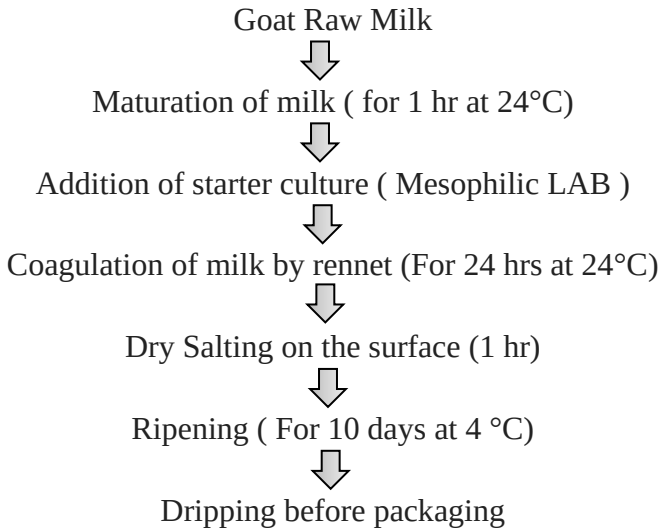
Appendix:

I. Table 3: Product details:

Product	Cheese
Name	Monte Enebro
Ingredient	Goat Milk ,Calf Rennet
Date of Purchase	12-APR-2021
Date of Expiry	26-APR-2021
Date of Examination	14-APR-2021

(Created by Author)

II. Figure 5: Flowchart of cheese production:



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III. Incubation time and temperature:

- Nutrient agar (NA) incubated at 25ºC (48-72 hrs)
- Violet red-bile-glucose agar (VRBG) incubated at 37ºC (24 h, no longer)
- de Man Rogosa and Sharpe agar (MRS) incubated anaerobically at 37 o C (48-72hrs)
- Baird Parker (BP) incubated at 37ºC for 24 hrs
- Listeria Selective Agar (LSA) incubated at 37ºC for 48 hrs (next week)

IV. Table 4: Colony counts after plating and incubation:

G-6	-1 (Dilution)	-2 (Dilution)	-3 (Dilution)
	Number of colonies identified		
VRBG	**$2.3*10^2$**	**$2*10^3$**	**0**
BPA	**TNTC**	**TNTC**	**0**
MRS	**TNTC**	**TNTC**	**TNTC**

G-5	-1 (Dilution)	-2 (Dilution)	-3 (Dilution)
	Number of colonies identified		
VRBG			
BPA			
MRS			

TNTC: Too numerous to count

(Created by Author)

V. Table 5: Result of gram staining:

Group Number	Name of cheese	Pasteurized / Unpasteurized	Media	Gram Staining	Cell Morphology
G-6	Monte Enebro	Unpasteurized	MRS	Gram +Ve	Short Rods
			BPA	Gram +Ve	Cocci Grape like structure
			LSA	Gram -Ve	Rods

(Created by Author)

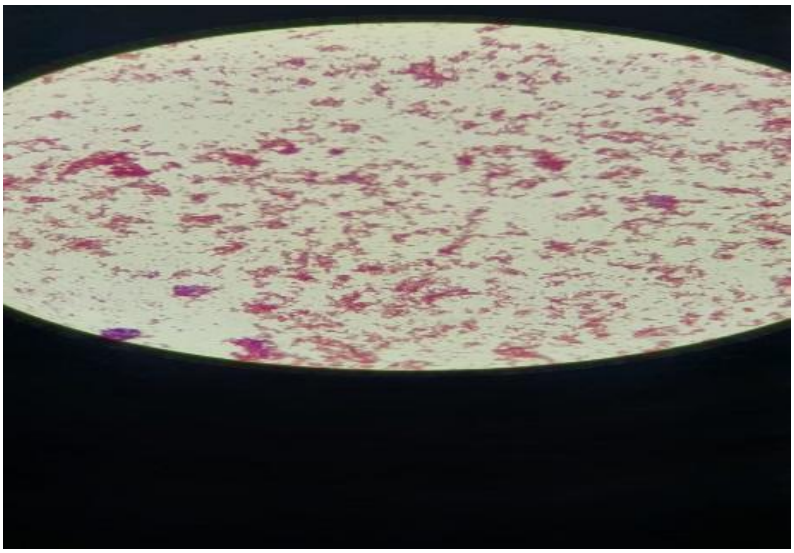


Figure 6: Gram staining of Colony from LSA

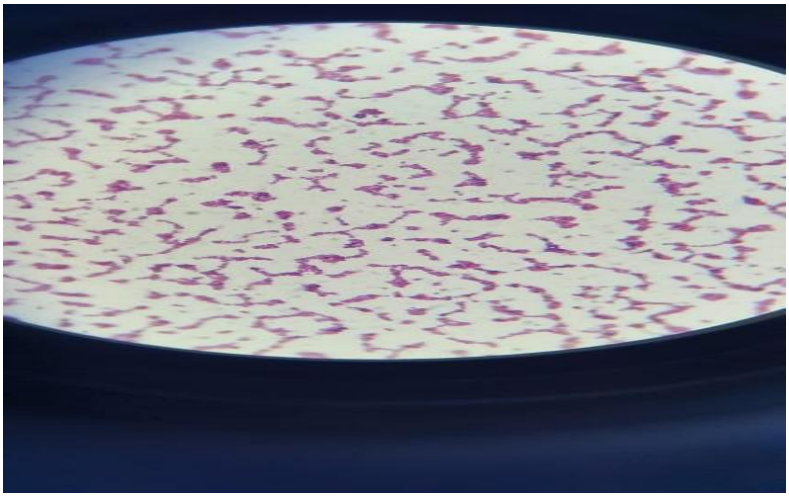


Figure 7: Gram staining of Colony from BPA

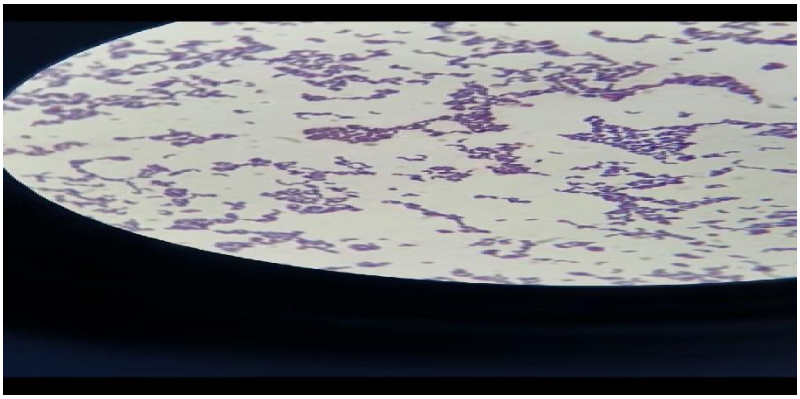


Figure 8: Gram staining of Colony of MRS

VI. Catalase test:

VRBG +ve , MRS -VE , BPA +ve

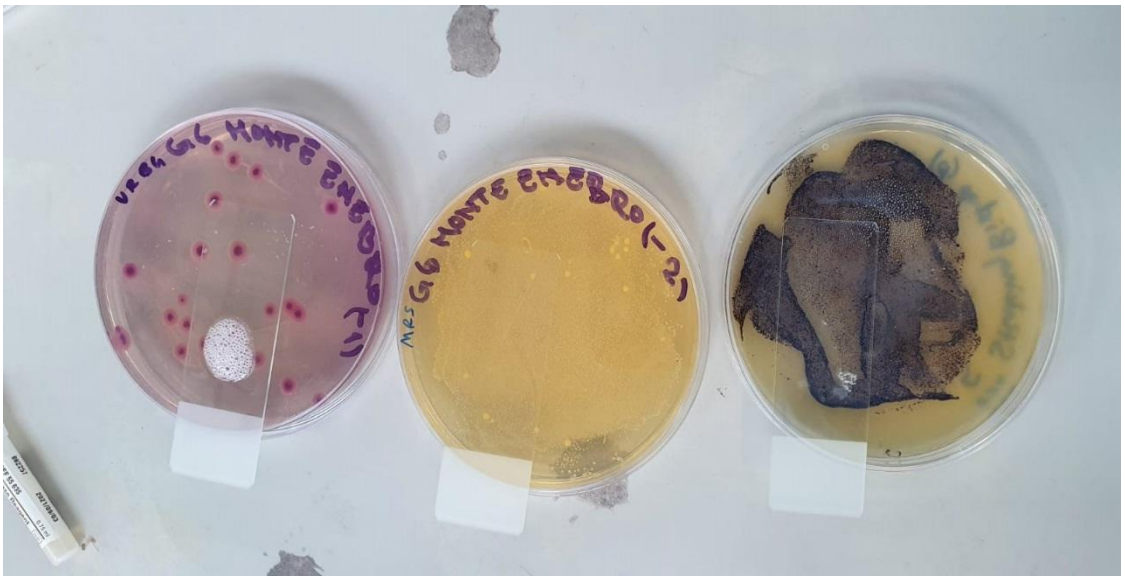
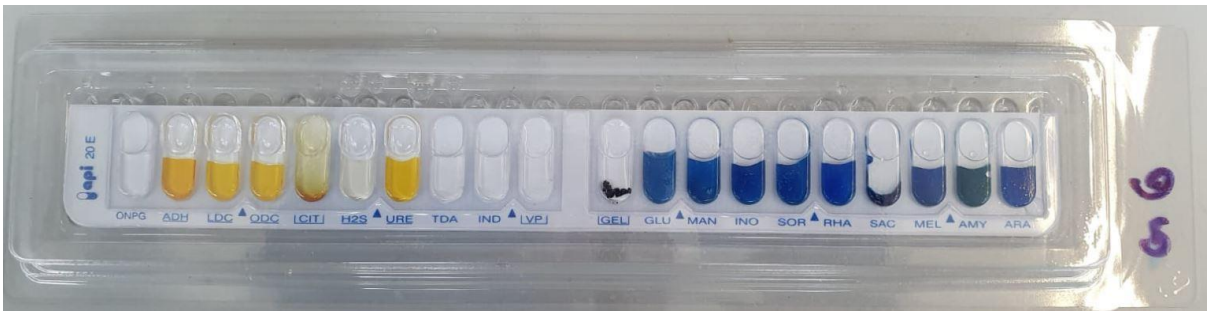


Figure 9: Catalase test

VII. API20E, API 50 CHL:

API20E:



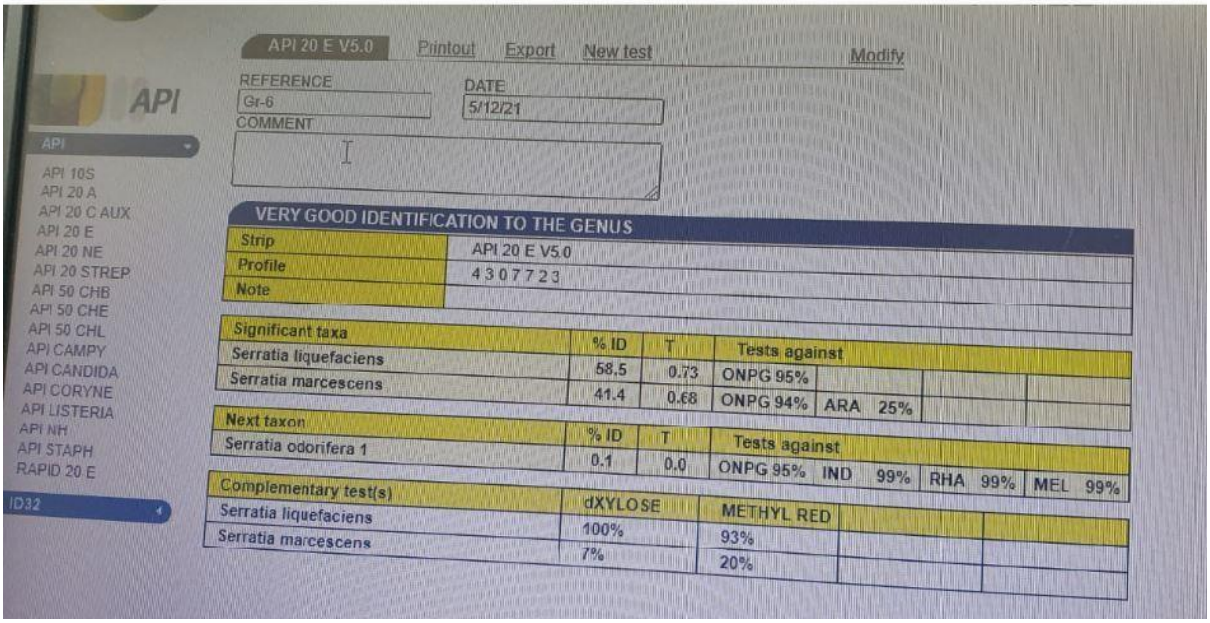


Figure 11: API20E

API50CHL:

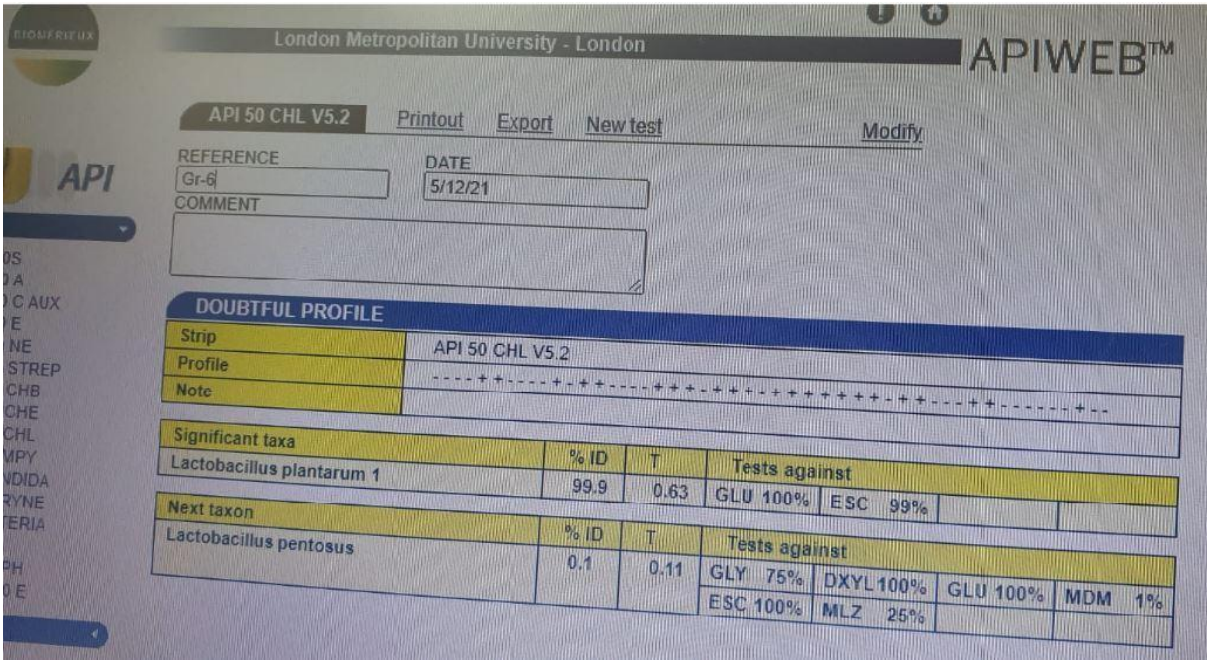


Figure 12: API 50 CHL

VIII. Result of blood agar:

BloodAgar:

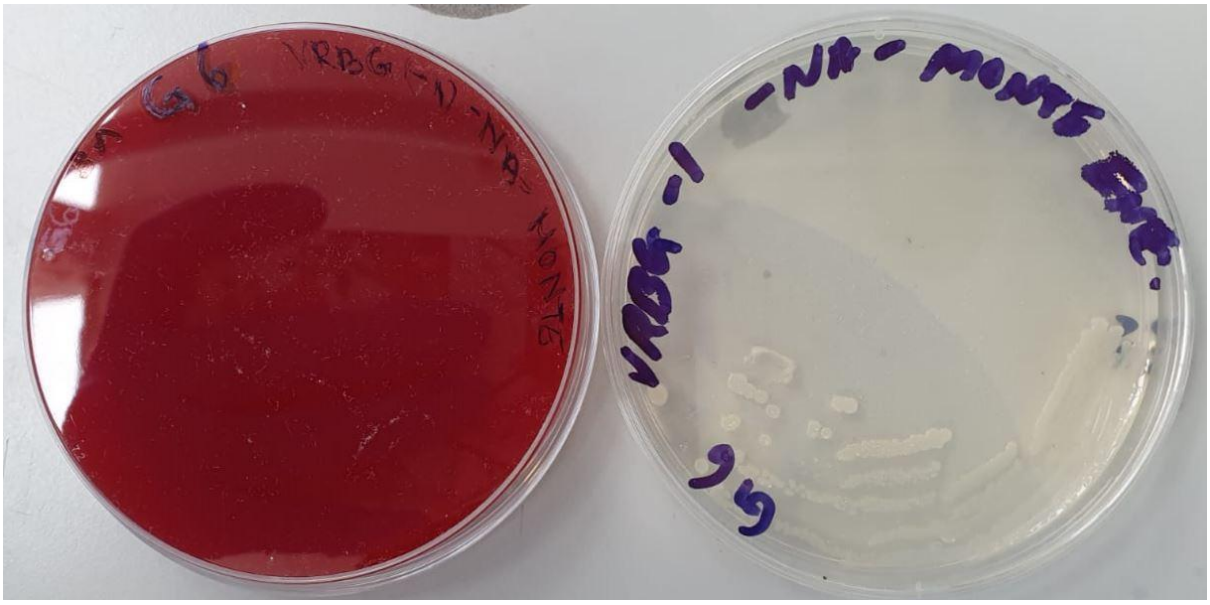


Figure 13: Colony taken from VRBG(-1)dilution on Nutrient Agar on right hand side and streaked out into left hand side blood agar petri dish.

Result of haemolysis after incubation:

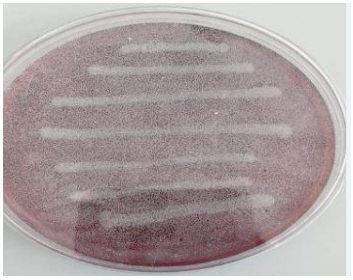


Figure 14: Haemolysis

IX. Staphylase Test or Coagulase test for *Staphylococcus aureus*:

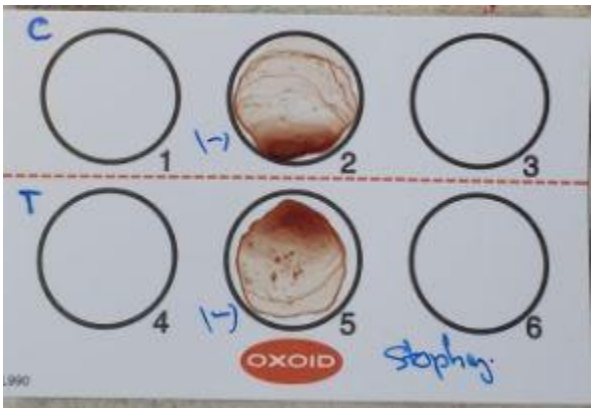


Figure 15: Staphylase test

X. *Salmonella* agglutination test:

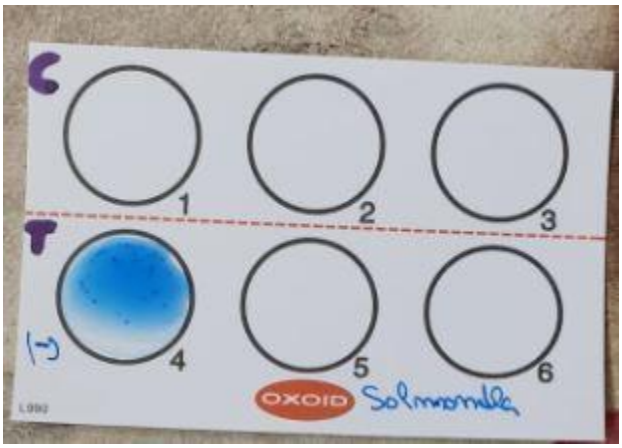


Figure 16: Salmonella test