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Effect of Temperature at the Shelf Life of Yogurt and the Role of *Listeria monocytogenes* and *E. coli* Inoculated in Unflavoured and Flavoured Yogurts

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Abstract: *E. coli* and *L. monocytogenes* are all dairy product related pathogens. The presence of these pathogens can lead to contamination. To increase the shelf life of yogurt we have to monitor the temperature at which it is stored, the change in microbial counts, pH, acidity, sensory attributes and percentage of free whey. Adaptation Test Acid demonstrates that the microorganisms such as Salmonella spp., *E. coli*, and L. monocytogenes are frequently identified to have a higher survival rate in meals when compared with non-adopted ones. When they are exposed to unfavorable growth circumstances such as severely acidic environments. In this study, we evaluated the survival of wild and adapted L. monocytogenes strains, inoculated at the same concentration around 4 log cfu/g; a slow decrease in the loads was observed until d 28 in unflavored inoculated with the wild strain**.** Three different experiments are performed on yogurt to evaluate the difference between flavored and unflavored yogurt, shelf life of them at different temperatures and to determine the role of *E. coli* and *Listeria monocytogenes* and see what changes it brings to the composition of yogurt. First trial is performed at three different temperatures. These are at 4, 8 and 20°C. Both flavored and unflavored yogurt sample showed low viable counts at 4°C until the end of trial. While performing this trial at 4°C the loads are lower in strawberry yogurts as compared to that of unflavored yogurt because potassium sorbate is present in fruit pure and anti-microbial activity is exerted by that. In second trial, *E. coli* and *Listeria monocytogenes* are added to the yogurt sample at two concentrations which 2 and 5 log cfu/g which will show a rapid decrease in acidic conditions in both flavored and unflavored yogurt samples. *Listeria monocytogenes* is very resistant in this case and presence of it can always be seen until the end period. In the third trial, the adaption of the yogurt sample is monitored after the inoculation of microorganisms in it. Between the wild acid adapted strains of *L. monocytogenes* no statistically significant difference is detected and that must be because to the quick adaption after the inoculation. Pasteurization is one of the main processes which are used to make the dairy products pathogen free and the basic functioning of it is through temperature and discussed in the paper.

Keywords: Yogurt, *E. coli*, *L. monocytogenes*, Shelf Life, Micro-organisms, Fermented Products

1. Introduction

Yogurt is a functional dairy product consumed worldwide because of its positive effect on human health. However, if the selection of raw materials is not well controlled, if good production practices are not used, especially in small-scale

production, or if storage conditions are unfavorable, yogurt spoilage occurs in a short time, leading to an unacceptable product for consumers due to its yeasty, fermented odor and flavor. Although yeasts are mainly known as positive organisms because of their fermentative activity for the production of several foodstuffs and beverages like wine, beer, and bread, they also play a major role as specific spoilage organisms and are the main cause of microbial alterations in yogurt. The ability to grow at low temperatures and in environments with high sugar concentration (up to 50–60%) allows yeasts to replicate in yogurt. Yeasts are generally more resistant than bacteria in extreme conditions, in particular thanks to low pH and the presence of preservatives [1]. The growth of yeasts on food components generates different end products, determining a substantial change in physical-chemical and sensorial characteristics. In particular, their ability to ferment lactose and sucrose, which are then converted to ethanol and carbon dioxide, leading to a distinctive alcoholic flavor, is well known. At the time of production, yogurt should contain no more than 1 yeast cell per gram, even if higher counts have been recorded immediately after production in any case, spoilage starts to be evident when loads reach 5 to 6 log cfu/g [2].

Yogurt has many positive effects on the human health that is why it is used worldwide as a functional group of our diet. Many dairy products like high protein yogurt and other fermented products have been used across the world in many different regions. In these regions more concentrated form use yogurt is been used while in the US region more liquid form of dairy products are usually used before the introduction of high protein yogurt [3]. Yogurt can also be a functional group of diet for the infants and for the sportsmen as well. Yogurt contains a high proportion of protein and fats which are very beneficial for a healthy diet [4]. The problem of cholesterol absorption can also be decreased by using the fermented products which contain probiotics. The major components present in yogurt such as protein, probiotics, calcium, lactic acid bacteria etc. can also help us in controlling blood pressure. It also corrects the digestion system and many researches also showed us that it also helps in the immune system of body [5]. In full fat milk 68 Kcal are present. There are many other non-nutrient components are also present in yogurt that act as an anti-cancer agent. The probiotics also reduce the allergenic potential of antigens. In 1780, first time the lactic acid was studied by Carl Wilhelm Scheele. Louis Pasture gave the idea that lactic acid can be formed by the fermentation of sugar by yeast. By using many starters cultures lactose in milk is converted into the lactic acid. This method was used long before the bacteriology is known. In 1921, Heineman stated the early method of yogurt formation which is by boiling milk in clean vessels at low heat. This will reduce its volume to one half from one fourth. Then, cooling at it 45-50°C and small amount of product from already existing lot will be added as well [6]. In modern techniques, appropriate fat contents and total solid contents are taken which are then homogenized, pasteurized and then are cooled at about 45-50°C. Then they are pumped into a vat and *Streptococcus salivarius* and *Lactobacillus delbrueckii* is added into it. Then the yogurt is stored and pH is maintained [7]. Sweetener such as sucrose can also be added into the yogurt to enhance the flavor or to make it more palatable. For this raw milk is heated at 60°C at sweetener is slowly added into it. After that the mixture is heated at 85°C for 10 minutes and it is cooled with ice water. After this the mixture is fermented and flavored yogurt is obtained [8]. Using different techniques, we analyze the nutrient content present in the yogurt. As the consumption of yogurt is increasing throughout the world people are now also conscious about the nutrient factors present in the yogurt. Microparticulate whey proteins are used which is also known as fat replacer. For this liquid milk is pre-heated at 60°C and powder ingredients are added to neutralize the yogurt dry matter. The yogurt mixture is then heated at 92°C for 10 minutes which will provide the optimal whey protein and then this is cooled at 42°C to add the yogurt culture [9].

2. Yogurt and Changes in the Shelf Life

To increase the shelf life of yogurt we have to monitor the temperature at which it is stored, the change in microbial counts, pH, acidity, sensory attributes and percentage of free whey. To evaluate the shelf life of yogurt, it will be stored at three different temperatures of 5°C, 15°C and 25°C. The sample stored at 5°C will be checked after every day, the sample stored at 15°C will be checked after every 16 hours while the sample stored at 25°C will be checked after every 8 hours for 3 days. By doing this microbiological and physiochemical analysis will be done. When we store the yogurt sample at three different temperatures one of them will definitely be more suitable than the other. If one of our samples has any sweetener or flavor in it that will can also effect the shelf of life of it because in this case it will be having different composition and we will be needing different temperature or environment to store it properly [10]. The end of shelf life of yogurt is usually determined by the sensory analysis. The changes in the sensory quality are determined by the changes in the specific attributes of yogurt or the change in the overall quality. The seasonal variations can also affect the quality of yogurt. For example, in some regions of the world the yogurt which is made from the mid-season milk has very least holding capacity with water while the yogurt made of late season milk has high holding capacity with water. The firmness of yogurt is also related with the gluconic-lactose induced gel. It predicts the seasonal changes of yogurt firmness [11].

3. Effect of *E. coli* and *L. monocytogenes* on Yogurt

Cell suspension of *L. monocytogenes* and phosphate buffer in it or we also add some other materials like glycerol, casein etc. These are stored at low temperature. This cell suspension is thawed at regular intervals and we can distinguish between

the surviving and non-surviving cells of *L. monocytogenes* [12]. Glycerol protects the *L. monocytogenes* from injury. In frozen state milk fat, casein and milk components provide it with protection but in long term glycerol is useful. If the death occurs more frequently than the milk fat ratio will increase as well. The *L. monocytogenes* can multiply in dairy products stored at very low temperature so we can also isolate *L. monocytogenes* from there. Freezing has both lethal and sublethal effects on *L. monocytogenes* which are basically dependent on speed of freezing, temperature of frozen storage, presence of nutrients and cycle of freezing [13].

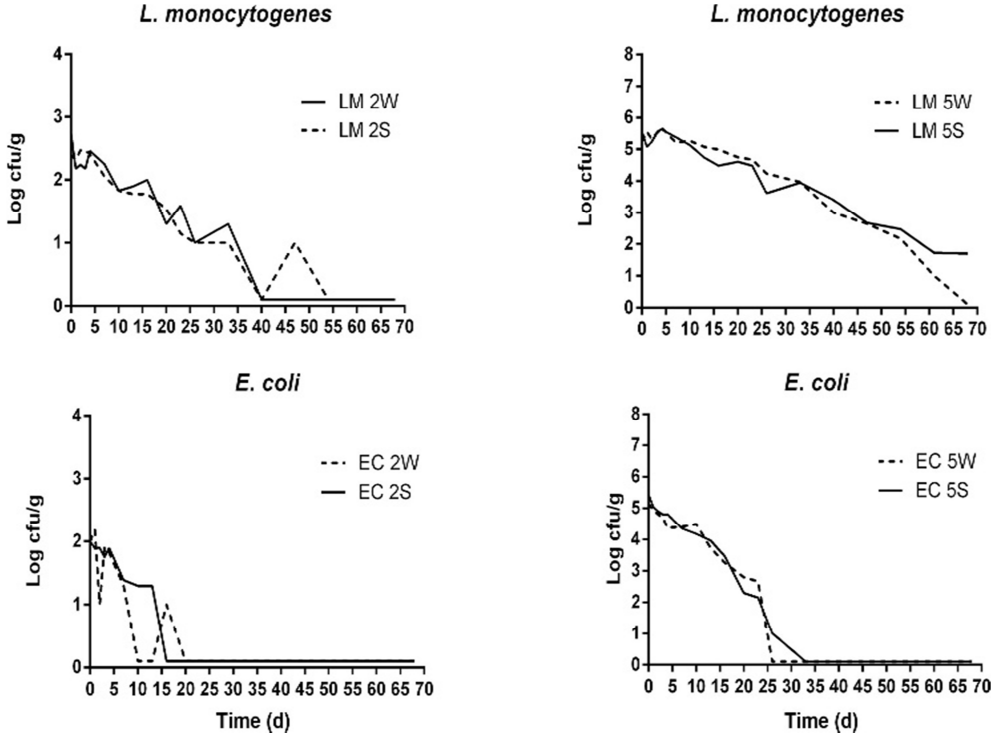

Figure 1. *Trends of Listeria monocytogenes and Escherichia coli counts in unflavored and strawberry yogurt samples inoculated at 2 different concentrations. (E. Tirloni, C. Bernardi, F. Colombo, S. Stella et al. 2015). https://doi.org/10.3168/jds.2015-9391.*

Inoculation Test was performed and the results obtained from the evaluation of the survival of *L. monocytogenes* and *E. coli* inoculated at 2 concentrations in the yogurt are shown in figure 1. We wanted to simulate the presence of these 2 microorganisms in yogurt to reflect postprocessing contamination rather than survival during production processes. Considering the samples inoculated with *E. coli* at a concentration of 2 log cfu/g, a rapid decrease in the loads was observed in both types of products; the results were below the detection limit (1 log cfu/g) at d 10 in unflavored samples and at d 16 in strawberry samples. Afterward, all the loads were below the detection limit, with the only exception of unflavored samples at d 16 where the concentration of 1 log cfu/g was counted. However, *E. coli* was present in 25 g of product until d 26 in unflavored samples, whereas it was present in strawberry samples until d 23. The figure describes 2W=unflavored (white) yogurt inoculated at 2 log cfu/g; 2S=strawberry yogurt inoculated at 2 log cfu/g; 5W=unflavored (white) yogurt inoculated at 5 log cfu/g; 5S=strawberry yogurt inoculated at 5 log cfu/g.

4. Incubation and Contamination

E. coli and *L. monocytogenes* are all dairy product related pathogens. The presence of these pathogens can lead to contamination, faculty pasteurization or poor hygiene handling by the handlers. For example, it can lead to soft cheese. One of the main losses of food in dairy industry is the contamination by the pathogens. In 2010, more than 10 million tons of dairy product wasted due to these pathogens [14]. The incubation temperature of *E. coli* is 37°C while the incubation temperature of *L. monocytogenes* is 6 and 37°C. The *L. monocytogenes* can be inhibited at a lower enzyme concentration. Heat treatment are the most reliable source of preserving the yogurt because all the pathogens including *E. coli* and *L. monocytogenes* are more lethal at low temperature and their growth can be nullified with high temperature. Ultra high pressure homogenizes is done to control the lethality *of L. monocytogenes* [15]. Maximum lethality of *L. monocytogenes* is in those dairy products in which the fat content is higher. *L. monocytogenes* and *E. coli* are pathogenic bacteria which are isolated from many of the dairy products. *E. coli* is a gram-negative strain and it produces Shiga like toxins that may cause abdominal cramps and diarrhea as well that can further lead to many other chronic diseases. On the other hand, *L. monocytogenes* is a gram-positive strain. It is structurally different from *E. coli* as well. Both of these pathogens have the ability to grow under

very harsh conditions and under a wide range of environmental stresses that may involve the fermentation to low pH [16]. Different studies about these pathogens have revealed that if we inoculate *the E. coli* and *L. monocytogenes* pathogens in different samples of yogurt and store each sample at different temperature than the sample which is stored at the very least temperature will survive because at low temperature the pathogens can grow better as compared to the higher temperature.

5. Role of Temperature in Diary Products

Temperature plays an important role to protect the dairy products from different kind of pathogens and its shelf life can be extended. The idea that the milk is stored by properly after heating or its quality can be maintained by heating is known even before the discovery of Pasteur when he showed that heating can inactivate the bacteria. In 1886, Robert Koch and many other health officers of UK discovered that the tuberculosis is mainly spread by using raw milk which is not properly stored. After this, in many regions of the world pasteurization technique was used to preserve the milk. The *Mycobacterium tuberculosis* was activated in the milk heated at 60°C for 15 minutes [17].

Temperature plays an important role in the manufacturing of yogurt. Temperature has an important role to play in the process of fermentation. All the raw products that are required to make the yogurt are mixed at a certain ratio to each other. While stirring, all the raw materials are mixed for 2h at the room temperature and then for rehydration it is stored overnight at 4°C. The yogurt is warmed at about 44°C for 1h before the heat treatment. The temperature at which the yogurt is treated is adjusted to heat the milk at 94°C for about 6 minutes and then quickly cooled at 10°C. After this they are stored at 4°C [18]. Temperature plays a key role in prolonging the shelf life of yogurt. The quality of yogurt is affected by all packaging, production, transport and storage. Through low temperature storage the shelf life yogurt can be enhanced. The number of starter culture of yogurt drops more when stored at a higher temperature. At 4°C, the number reduced by 2 logs cycle every month but at 30°C this number is 10 log cycles. The sample stored at 30°C can be reformed to yogurt after 1 month but if the same sample stored at 4°C than can reformed even after 3 months. In this way, the shelf life is prolonged [19]. To distinguish between the life of microorganisms and other raw materials of yogurt we will store our yogurt sample at three different temperatures which will be 4°C, 8°C, 20°C. We will see that at 4°C the loads will 9.0 to 8.8 log cfu/g, at 8°C this value will be 8.9 to 8.6 log cfu/g. At both of these temperatures almost similar trends will be registered. While the sample stored at 20°C will give the value of 8.2 to 7.6 log cfu/g. There will be an exception here. This is because most of the pathogens that affect the yogurt can survive at higher temperature but they cannot survive at lower temperature [18].

Table 1. *Presence in 25 g of Listeria monocytogenes (LM) in wild or adapted in unflavored (white; W).*

Item[1]	0	7	14	21	28	35	42	49	56	63	70
LM wild											
W	+	+	+	+	+	+	+	+	−	−	−
W	+	+	+	+	+	+	+	+	−	−	−
LM adapted											
W	+	+	+	+	+	+	+	+	−	−	−
W	+	+	+	+	+	+	+	+	+	−	−

Sampling time (day)

[1]Wild=strain not adapted; adapted=strain isolated from yogurt at T68 of the inoculation test.

Adaptation Test Acid demonstrates that the microorganisms such as Salmonella spp., *E. coli*, and L. monocytogenes are frequently identified to have a higher survival rate in meals when compared with non-adopted ones (defined also as wild). Furthermore, when exposed to unfavorable growth circumstances such as severely acidic environments, L. monocytogenes respond by growing the synthesis of important virulence components. In this study, we evaluated the survival of wild and adapted L. monocytogenes strains, inoculated at the same concentration around 4 log cfu/g; a slow decrease in the loads was observed until d 28 in unflavored inoculated with the wild strain**.** Following that, the loads were below the detection limit (1 log cfu/g). A comparable decline was found in samples infected with the adapted strain, with measurable numbers in unflavored samples until d 28. However, L. monocytogenes was found in the product until d 56 in adapted samples and d 63 in wild samples. Throughout the study, no statistically significant changes were found between the wild and adapted strains. This might be attributed to L. monocytogenes rapidly adapting to environmental circumstances typified by low but not inactivating pH values between 4.1 and 4.3, allowing for greater cell survival during the whole period studied. L. monocytogenes tested in different combined stress conditions and initially adapted to slightly acid conditions have not shown a greater reduction in lag phase or higher average daily gain compared to the wild strain, confirming a very rapid adaptation of the strains in a new mildly acidic substrate.

6. Pasteurization of Dairy Products

Pasteurization is one of the main processes which are used to make the dairy products pathogen free and the basic functioning of it is through temperature. We treat our dairy product with temperature at 72°C for 15 seconds so in this way the pathogens are killed and the dairy product can be consumable for a longer period of time. After this inoculation of 2-3% starter culture is done and then we will incubate it for 2 to 3 hours at 40 to 45°C [20]. Fresh yogurt is fortified with the lactic acid bacteria and is prepared in two different sets and is supplemented with the butter milk powder. All these samples were sprayed dried through two different inlets whose temperature is being set at 120°C and 150°C while the outlet temperature is fixed at 60°C. The survival of *L.*

helveticus is evaluated through this process using survival percentage and bacterial count. After 3 months the result indicated that *L. helveticus* can survive in the powder obtain through the spray drying at 120°C. *L. helveticus* is a lactic acid producing bacteria which is used in the preparation of many dairy products [21].

Yeast spoilage causes a huge economic damage to the dairy industry because a lot of products is being wasted due to it. A bioprotective system is made which will give the predictions of yeast spoilage in yogurt. Growth characterizations of *Debaryomyces hansenii*, *Yarrowia loplolytica, Sccharomyces cerevisiae and Kluyveromyces marrinus* are conducted in yogurt at temperatures of 7, 12 and 16°C for a storage period of time which will be 30 days. These will be without bioprotective culture. Monte Carlo model is used to translate the level of yeast growth into the spoilage level. By comparing the output of these models and the actual data the models are validated [22].

7. Difference Between Flavored and Unflavored Yogurt

To store the yogurt for a longer period of time we must adjust the certain pH of it which is 4.4. In flavored yogurt commercial fruit flavorings are added to enhance the sweetening of yogurt base. These sweeteners are usually sucrose, dextrose and fructose. Sweeteners are used at different combinations by weight. During the incubation and after packaging the evaluations are done. The evaluation has shown that the amount of sweetener has increase the time require to attain the 4.4 pH and this time is significantly shorter for those samples which do not contain the sweeteners. This difference is due to the presence of more solids instead of the sweeteners [23]. An analysis is done to determine that whether the liking or disliking of the consumer of yogurt depend on the sweeteners or not. For this 19 samples of yogurt are taken out of which 14 are strawberry flavored, 12 are raspberry flavored, 6 are lemon flavored and 17 are unflavored. Different people are being added onto the panels to determine any preferences. The titratable acidity and pH of all the samples is measured. The overall liking of the consumers is highly correlated to the intensity of sweetness, sourness: sweetness ratio. In general, it is found out that higher the sweetness, higher the acceptance of yogurt in consumers [24].

Monk fruit extract (MFE) is used as a sweetener in place of sucrose. Yogurt sweetened by MFE will not be having extra sugar calories in it. The microstructural properties of yogurt sweetened by MFE are the same of the yogurt sweetened by the sucrose. The levels of Gly-Pro p-nitroanilide hydrochloride and dipeptidyl peptidase IV inhibitory activities, 1,1-diphenyl-2-picrylhydrazyl radical and superoxide anion radical scavenging ability are higher than compared to other yogurt samples. MFE can be a novel sweetener for yogurt and other dairy products [8]. If the amount of acetaldehyde, lactic acid and acetic acid which are the desirable end products of fermentation is not optimum than the yogurt will have atypical or weak flavor. If excessive number of starters or *L. bulgaricus* predominates are used than a harsh acid flavor will occur. The overproduction of acetaldehyde can also produce a harsh flavor. In yogurt aroma, the most important component is acetaldehyde. If the value of acetaldehyde is less than 4.0 ppm than weak flavor is observed while if the value of acetaldehyde is higher than 8.0 ppm than string flavor is observed [25].

Most often, sucrose is used to sweeten the yogurt. After the flavoring agents are added in the yogurt, it will be containing 0 to 18% of sucrose. Sucrose is also used to sweeten the unflavored yogurt. This is because most of the manufacturers believe that the consumers prefer a slightly sweetened yogurt. Also, if the plain yogurt is sweetened than it will contribute in the manufacture of all other desired flavored yogurt. Sugar also contributes in many physical and chemical properties of dairy products [26]. The sweetener that is being used to add the flavor in the yogurt can also be traced. This process is done by using linear discriminant analysis (LDA). By inoculating lactic acid culture unto the milk yogurt is made and three different sweeteners are used which are sucrose, honey and sucralose. Physiochemical and sensory properties of yogurt are also analyzed. The yogurt sample with honey has higher protein content. The yogurt sample with sweeteners in it has lower fat content present in it. The overall sensory result showed that the yogurt sample with honey in it can attain the acceptable quality upon processing [27].

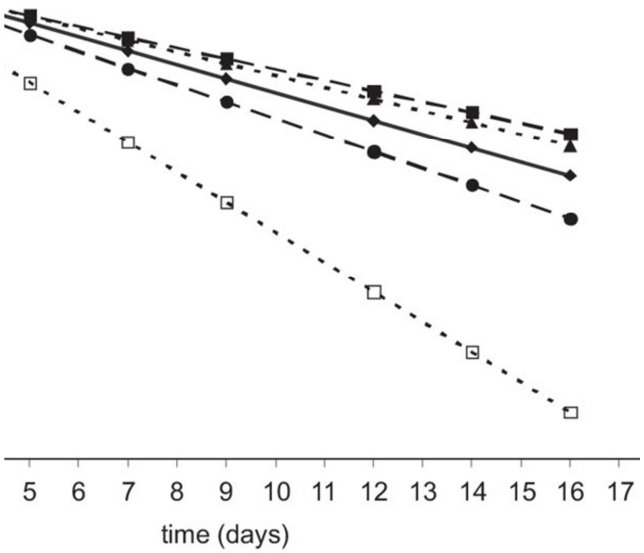

Figure 2. *Survival of Listeria monocytogenes in yogurt stored at different temperatures. (J. Szczawiński et al). DOI 10.1515/pjvs-2016-0039.*

8. Conclusion

L. monocytogenes can also help in the production of many dairy products. The strains of *L. monocytogenes* are taken from the stock culture and are then transferred to the tryptose broth and are incubated at 35°C for 24 hours. Another

transfer is made to tryptose broth and incubates it as well as described above. Then, transference is made of *L. monocytogenes* from the last culture into 200ml sterile skim milk and incubation is done at 35°C for 48 hours. An amount of this culture will be sufficient to give us the product, is diluted with sterile 2% citrate solution and these contents are added into our dairy product. The starter cultures, *Streptococcus thermophilus* and *Lactobacillus bulgaricus*, are taken and are transferred into the dairy product and incubate it for 14 hours at 37°C [28]. The pasteurized and raw milk is inoculated with the bioluminescent strains of *L. monocytogenes* and *E. coli*. These are used in the production of dairy products. The survival of both of these microorganisms is evaluated during the manufacturing and storage stage. Bacterial bioluminescent is used to indicate the colonies of these microorganisms. E. coli survive the manufacturing process and are present in higher number at the end of the process than the initial count. The number of *L. monocytogenes* increase with the increase in the number of the pH [13]. Nisin is a bacteriocins and it is produced by the strains of lactic acid bacteria and they are effective in inhibiting the gram-positive bacteria including *L. monocytogenes*. It is available commercially and is permitted to be used for many applications in food. The yogurt produced from the camel milk has many therapeutic properties because camel milk can suppress the growth of many foodborne pathogens. Inoculate *L. monocytogenes* and *E. coli* in this yogurt to check the effect of it in the yogurt. Both of these are inoculated in the yogurt and fermented at 43°C for 5 hours. We will use freeze-dried lactic acid bacteria starter culture and then store it at 4 or 10°C for 14 days. Another sample is also prepared which is without the lactic acid bacteria starter culture. During the process of fermentation, the number of both *L. monocytogenes* and *E. coli* will increase both in the presence and absence of lactic acid bacteria. In storage the number of *L. monocytogenes* and *E. coli* will increase in the absence of lactic acid bacteria but in the presence of lactic acid bacteria the number of both of them will decrease. *E. coli* will not be detected after 7 days of storage in the yogurt made with lactic acid bacteria. On the other hand, *L. monocytogenes* will still be able to tolerate it at the refrigerator temperature [16].

9. Future Possibilities

A study is aimed to evaluate the behavior of *L. innocua* in a dairy desert in the absence and presence of probiotic microorganisms. Three formulations of desert dairy are made, first is inoculated with *L. acidophilus*, second is inoculated with *L. innocua* and third is inoculated with both of L. *acidophilus* and *L. innocua*. These formations will be stored at 28°C for 5 days. The pH and numeration of microorganisms is determined at days 0, 7, 14, 21 and 28. The result shows us that the pH of formulation inoculated with *L. acidophilus* has decreased at the end of the shelf life, while the pH of other two formulations has increased. The counts of *L. acidophilus* also decreased throughout the shelf while the count in other formulations has increased. The results of this study show the needs to ensure the microbiological safety of raw materials, manage the pasteurization temperature and to ensure that surfaces and environments are cleaned and sanitized to avoid contamination by any pathogen and particularly, by *Listeria monocytogenes* [29]. Yogurt stored at 4°C was very stable and under conditions of heat stress, yeast growth was a limiting factor in microbiological shelf life. Potassium sorbate in strawberry yogurt had antimicrobial action and is considered one of the protective properties of fruit yogurts. The natural acid conditions provided by the products allow the survival of *E. coli* and *especially L. monocytogenes* in the samples from both inoculum bases. If different samples of yogurt inoculated with *E. coli* and *L. monocytogenes* and are stored at different temperatures, then the sample stored at the very least temperature will survive the best because pathogens can grow better at low temperature. Strong acid resistance resistant to *L. Monocytogenes* have led to the formation of organic bacteria in both types of yogurts all the time being observed, by rapidly adapting to the intermediate substrate. The natural persistence of this virus is a major concern for professional dairy producers and should be considered in risk assessment, especially in low-value areas.

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