Evaluation of the Potential Lactic Acid Bacteria (LAB) to Ferment Quinoa Milk

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Chandana Jois

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ABSTRACT

The study aimed at (a) investigated the ability of potential lactic acid bacteria (LAB) to ferment quinoa milk, and (b) formulating a method for producing fermented quinoa milk. To meet the objectives, *Lactobacillus pentosus* and *Pediococcus pentosaceus* two native bacterial strains were tested. Quinoa milk elaboration was optimized at laboratory scale by selecting an appropriate quinoa/water proportion. Induced fermentation process was performed using the mentioned bacterial strains individually as starter cultures on the quinoa milk. Enumeration of viable cells was done on different nutrients agar to characterize the fermented quinoa milk microbiota. After fermentation, the stability during storage time was followed during 28 days at 4°C. It was monitored the pH, lactic acid concentration, and survivability of the starter cultures. After 48 hours of fermentation, there was a statistically significant decrease in pH and increase in lactic acid. During storage time, the pH and lactic acid content remained relatively constant with no statistically significant difference ($p < 0.05$). This study confirms that the two bacterial strains can be used as starer cultures to ferment the quinoa milk. *L. pentosus* had the higher survivability rate during storage compared to *P. pentosaceous*. The finding reveals that better results were obtained using *L. pentosus*. The fermented quinoa milk contained between $2.6x10^7$ CFU/ml to $5.9x10^8$ CFU/ml for *P. pentosaceus* and $4.2x10^7$ CFU/ml to $3.8x10^8$ CFU/ml for *L. pentosus*, therefore the fermented quinoa milk can be considered as a probiotic drink.

AIMS

The main aim of this study was to evaluate the ability of the potential lactic acid bacteria (LAB) to ferment the quinoa milk. This study also aims at formulating and optimizing the process for producing fermented quinoa milk, investigate and monitor the microbial survivability of lactic acid bacteria, pH (including fermentation time and temperature requirement to achieve the desired pH), acidity and shelf life.

1. BACKGROUND

1.1. Quinoa grains

In recent years, quality and health are two essential factors that is gaining importance, henceforth, development of new products with good qualities and health benefits are increasing significantly (Andrea et al, 2013). In this regard, cereal grains gain focus since they are staple foods with significant effect on health and evidently form a part of human diet (Silvana et al, 2016). Eventually, there has been an increasing research on alternative grains due to the fact that consumers prefer natural products with health benefits (Silvana et al, 2016). One alternative is quinoa grains.

Quinoa is a pseudo-cereal which is mainly grown and cultivated in the Andean region of South America. Archaeological findings reveal that Quinoa was cultivated before 5000 BC (Jancurova et al, 2009). Botanically, quinoa belongs to *Chenopodiaceae* family and *Chenopodium* genus. Quinoa can be cultivated in the altitudes from sea level to Andean highlands. Quinoa has the capacity to withstand wide range of pH even in the salty soil ranging from 6.0 to 8.5 pH. Quinoa is temperature tolerant and it is said to be frost resistant ranging from 0°C upto 35°C and it is also drought resistant (Jancurova et al, 2009). Recently, quinoa has been introduced in Europe, Asia, Africa and North America (Jacobsen, 2003).

Quinoa grains has gained increased attention because of its high nutritive values such as protein, carbohydrates, fibre, vitamins, minerals and low-fat content compared to other cereals (Matsuo, 2015) (Gordillo et al, 2016). The dietary fibres consist of approximately 2.6%-10% of the total weight on the quinoa grain (Yao et al, 2014). All ten essentials amino acids are present in quinoa therefore quinoa fulfils the amino acid requirements for adults and can serve as a valuable source of nutrition for infants and children (Gordillo et al, 2016). Studies reveal that polysaccharides in quinoa have antioxidant properties (Yao et al, 2014) (Graf et al, 2015). Quinoa grains are rich in vitamins, minerals, polyphenols and Betaine. Henceforth, Quinoa is an excellent gluten-free grain, and is potentially a part of healthy diet (Graf et al, 2015).

Quinoa can be considered as a healthy, alternatively for people suffering from celiac disease. Celiac disease is an immune mediated reaction exacerbated by taking grains containing gluten (Saturni, 2010). Celiac disease is said to affect one percent of the population in Europe and in United States and a strict gluten free diet is prescribed as the only treatment (Jnawali, 2016)

and therefore there is huge demand for gluten free products. Studies reveal that quinoa contains many useful vitamins, minerals and additional compounds and hence it can be used to control type 2 diabetes and improve metabolic risk factor profile (Yao et al, 2014).

There are other substituents to dairy milk in the supermarket that are made from cereals, seed, legumes, nut and pseudo-cereal. But these substituents have certain drawbacks including allergic reactions and the presence of gluten, higher glycemic index and higher fat content (Pineli et al, 2015) (Graf et al, 2015). With this regard, quinoa has low fat content and lower glycemic index as compared to other cereals and other existing non-dairy milk thereby reducing the risk of any allergies, reactions or intolerance (Pineli et al, 2015).

Quinoa grains is chosen for making vegetable milk since it has all the above-mentioned health benefits specially gluten free, and quinoa is said to be a great crop alternative to solve food shortages (Urquiso et al, 2017). Fermentation technique is carried out to obtain the desired product. Fermentation is one of the ancient techniques used for preserving food, also it helps in improving the functionality, flavour and texture of food products (Hill et al, 2017). Also, fermentation is a cost-effective technique which increases iron solubility of quinoa (Valencia et al, 1999). Fermented vegetable milk has health benefits like improving the digestive system, hence fosters the digestion process and fermented vegetable milk is rich in nutritional composition and reduces risk of diabetes, fights cholesterol and body fat (Maria, 2017).

Therefore, fermentation-based quinoa milk is opted in order to improve the product quality, shelf life and ultimately to fulfil the raising demand of the market with allergen free product and can be considered as a cost effective, healthy drink.

1.2. Fermentation

Fermentation is an ancient technique that is used to preserve by the action of microorganisms. Main action occurs by the conversion of carbohydrates to alcohol and organic acids (FA0, 1998). There are two types of fermentation which includes aerobic fermentation (in the presence of oxygen) and anaerobic fermentation (in the absence of oxygen). Fermentation of food covers a range of enzymatic and microbial processing of food and food ingredients in order to attain desirable features like increasing shelf life, health promotion, enhanced flavour and nutritional enrichment and improved food safety (Giraffa, 2004). Fermentation leads to the production of lactic acid, which helps in decreasing the pH (Liu et al, 2009).

Microbiology properties and chemical characteristics often have an important impact on the fermented quinoa milk. Particularly, microorganisms are used within fermentation industry for the production of specific metabolites like acids, antibiotics, carbohydrates and enzymes. microorganisms play a predominant role in this process in providing required acidity to the quinoa milk (Giraffa, 2004). Microbial techniques are important in order to analyze the activities of the bacteria in the fermented quinoa milk (Giraffa, 2004) (Urquizo et al, 2016). Food fermentation is necessary in ensuring the microbiological safety and shelf life of the product (Caplice et al, 1999). Lactic acid fermentation has been studied for several years; they help in reducing the acidity of the product (generally below pH 4) by producing organic acids. This reaction typically can be used for fermentation of cereal grains and to add on they help in enhancing the nutritional content in the product and improves digestibility (Karovicova, 2007). Lactic acid bacteria play a predominant role in fermentation as they induce texture and flavour changes and produces a preservative effect (Liu et al, 2009). Fermentation of food products helps in enhancing the organoleptic characters and provides food safety by inhibiting the growth of harmful bacterium (Urquizo et al, 2016).

1.3. Lactic Acid Bacteria (LAB)

Lactic Acid Bacteria is a type of microorganisms that has the ability to ferment glucose to lactic acid or lactic acid, carbon-di-oxide and ethanol as an end product of fermentation. The term Lactic Acid Bacteria is reserved for genera in the order *Lactobacillales*, that includes *Leuconostoc*, *Pediococcus*, *Lactobacillus*, *Lactococcus* and *Streptococcus*. Lactic Acid bacteria lowers the pH of the fermented food to below pH 4. They are regarded as an important group of microorganisms used in fermentation of food and LAB enhances taste and texture of fermented products. Additionally, LAB prevents food from food spoilage bacteria by producing large quantities of lactic acid and growth-inhibiting substances and thereby LAB helps in preserving the quality of the food (Todar, 2008).

Probiotics is defined as 'live microorganisms which when administered in adequate amount confer health benefits to the host' (FAO/WHO, 2002). LAB is also regarded as a probiotic due to its characteristics features of enhancing health benefits. LAB strains produce antimicrobial substances, with activity against the homologous strain (Ljungh, 2006). LAB reduces the risk of gastrointestinal (GI) infections and in certain cases, they help in treating the GI infections (Nagpal et al, 2012). In order to have health benefits and to be considered as a probiotic product, food should consist of 10^6 CFU/g of viable probiotic bacteria (Kechagia et al, 2013). Studies

reveal that consumption of probiotic (ranging from 10^9 -10^{10} CFU/g) per pay provides significant health benefits. However, consuming probiotics on a regular basis is considered to enhance health benefits (Kechagia et al, 2013).

1.4. *Lactobacillus pentosus*

Lactobacillus pentosus is a type of lactic acid bacterium which can be used as starter culture for enhancing the process of fermentation. Studies reveal that *Lactobacillus pentosus* have probiotic effects of reducing the growth of spoilage bacteria and thereby inhibiting bacterial infections. They also have potential to improve the mucosal immunity and promotes health benefits (Barragan et al, 2011). *Lactobacillus pentosus* has also demonstrated good growth and increased capacity to survive under stimulated gastro intestinal environment and has the potential to aggregate and co-aggregate with harmful bacterium, adhering to intestinal cell line and has antagonistic activities against the pathogenic bacteria and has the ability to survive and render health benefits (Abriouel et al, 2017) (Montoro et al, 2016).

1.5. *Pediococcus pentosaceus*

Pediococcus pentosaceus is a member of Lactic acid bacteria. It belongs to the genus *Pediococci* and they are acid tolerant and have a fermentative metabolism producing lactic acid as the metabolic end product. *P. pentosaceus* regulates the growth of food borne spoilage bacteria. Studies reveals that cultures of *P. pentosaceus* has the potential to inhibit Listeria proliferation (Jang et al, 2015). *P. pentosaceus* can survive in the gastric and intestine conditions (acidic environment) and pH by stimulating the immune activity (Jonganurakkun et al, 2008).

2. MATERIALS AND METHODS

2.1. Formulation of Quinoa Milk – Pre-Trials

Quinoa milk was prepared in the food grade lab at Kemicentrum, Lund. Parameters considered was washing, toasting time, temperature, mixing rate, filtration, and dilution. Other sensory parameters considered was taste, flavour, colour, and aroma.

In the Pre-trials, varying amount of quinoa grains was used to determine the appropriate quantity required for preparing the quinoa milk. 50g of raw quinoa grains (white quinoa) was washed with distilled water in the ratio 1:2 (w/v) with varying duration of time. Washing time and method includes washing the quinoa grains under running water, washing for 3 minutes,

soaking the grains for 1 hour, 12 hours and 15 minutes. Washed quinoa grains were then dried on the sieve and transferred onto the frying pan. Different toasting temperature and time was performed to identify the ideal time and temperature required to obtain the desired properties of the quinoa milk which includes nutty flavour and golden coloured grains (Table 1). The roasted quinoa was mixing with autoclaved water proportionally 1:4 (w/v) using a blender at different speed settings (rpm). The mixture was then filtered through a metal sieve of pore size 250 m and collected into glass bottles (550ml), stored in 4°C for further analysis.

Table 1: Different trials performed for formulating the quinoa milk with different heat setting, toasting time and filtration method

Sample No.	Weight of quinoa grains (g)	Heat Setting	Toasting time (minutes)	Method of filtration
A	50	7	15	Double filtration
B	50	6	10	Double filtration
C	50	5	20	Double filtration
D	50	4	20	Double filtration
E	50	5	12	Single filtration
F	50	7	5	Single filtration
G	50	8	4	Single filtration

2.1.2. Preparation of quinoa milk

Based on the trials (section 2.1), it was observed that the optimum parameters were to follow the preparation of sample D. The proportions of quinoa/water (Autoclaved milli-q water) were modified to 1:8 (w/v) and mixed using a blender for about 4 minutes at setting 2 (rpm) to ensured that the grains are well mixed with the water. After that, the mixture was then filtered using a cheese cloth and distributed into glass bottles. Thereafter, the quinoa milk was stored at 4°C overnight before inoculation and fermentation. Throughout the procedure ethanol was used to clean the equipment and gloves were worn to avoid any possible contamination.

2.2. Reactivation of Bacteria

Two bacterial strains *Lactobacillus pentosus* (marked as 2) and *Pediococcus pentosaceus* (market as 5) were used for carrying out the fermentation process and for microbial analysis. The cell concentration was measured on 1 ml of 0.085% NaCl solution, followed by serial dilutions, and absorbance was measured on the UV Spectrophotometer at 610 nm by duplicate.

2.3. Fermentation Process

Induced fermentation process was carried out for the quinoa milk. Six bottles (550ml) were filled with quinoa milk and inoculated separately with *L. pentosus* 2, and *P. pentosaceous* 5. The bottles were marked as 1, 2, 3, 4, 5 and 6, and incubated at 30°C.

After fermentation, the bottles were opened and used for microbial analysis, pH and acidity. All the bottles were then stored at 4°C for 28 days. Microbial analysis, pH and acidity was monitored during storage on 14^{th} and 28^{th} day.

2.4. Measurement of pH

Before sampling, the pH was measured using the pH meter (KEBO lab 744 pH meter, Metrohm).

2.5. Microbial count (viability) of the fermented quinoa milk

Microbial viable cells count was carried out before fermentation process (marked as 0 hours) after 2 days of incubation (marked as 48 hours) and during storage time on the 14^{th} (marked as 14 Days) and 28^{th} days (marked as 28 Days). Volumes of 10ml of the quinoa milk was mixed with 90ml bacteriological peptone water (peptone water is used as a microbial growth medium which is composed of peptic digest of animal tissue and sodium chloride) and vortex. Serial dilution was performed for the above sample and 0.1 ml of the sample was transferred on to the plates (TSA, ROGOSA, VRBD and MALT) and spread evenly in duplicates. VRBD plates were incubated at 37°C for 24 hours. ROGOSA plates were incubated at 37°C for 72 hours in an anaerobic condition. TSA plates were incubated at 30°C for 72 hours and MALT was incubated at room temperature for 7 days. Two colonies randomly were chosen from the and streaked onto fresh agar plates and incubated as mentioned above.

2.6. DNA Extraction

Purified bacteria was transferred onto 1ml eppendorf tube containing 500µl of autoclaved mili-Q water and 8-10 glass beads. The tubes were then transferred to the shaker and homogenized for 45 minutes. Later on, the tubes were centrifuged at high speed at 14,700 rpm for 1 minute and stored at -4°C for further use.

2.7. 16S rDNA Polymerase Chain Reaction (PCR)

16S rDNA PCR reaction was carried out to perform the amplification of the genes from the extracted DNA samples in which the nucleotides sequences would be used to identify the bacterial isolates by performing 16S rRNA sequencing. PCR reaction was initialized by suspending 2.5µL of extracted DNA samples with 22.5µL of PCR Master mix. The PCR Master mix contains 18.375µL nuclease free water (Qiagen, Germany), 2.5µL Top *Taq* buffer (Qiagen, Germany), 0.5µL dNTP mix (Qiagen, Germany), 0.5µL ENV1 served as forward primer (5’ – AGA GTT TGA TII TGG CTC AG – 3’), and ENV2 as reversed primer

(5’ – CGG ITA CCT TGT TAC GAC TT -3’) (Eurofins Genomics, Germany) and 0.1255µL Top *Taq* polymerase (Qiagen, Germany). The entire contents of the PCR Master mix were homogenized by using vortex for 30 seconds. 22.55µL of PCR Master mix was transferred into PCR tubes. Amplification of genes were performed at 94°C for 3 minutes, denaturation at 94°C for 30 seconds, then annealing at 60°C for 30 seconds and extension at 72°C for approximately 1 minute (TopTaq PCR Handbook, 2010). The reaction was confirmed by gel electrophoresis.

2.8. Random Amplified Polymorphic DNA (RAPD)

The RAPD procedure was followed according to Quednau (1998). Briefly, the reaction was initialized by suspending 2µL of extracted DNA and 48µL of Master mix. The RAPD Master mix contains 39.5µL of nuclease free water (Qiagen, Germany), 5µL of Top *Taq* buffer (Qiagen, Germany), 1µL dNTP mix (Qiagen, Germany), 2µL of P-73 as primer (5’-ACG CGC CCT-3’) (Eurofins Genomics, Germany) and 0.5µL Top *Taq* polymerase (Qiagen, Germany). The RAPD product were confirmed by using gel electrophoresis to observe the amplification.

2.9. Gel Electrophoresis

Gel electrophoresis was carried out in order to observe the amplification from the PCR and RAPD products. A solution of 1.5 % of Agarose (pure grade powder, Electran, VWR, Belgium) was used. The electrophoresis was carried out mixing 2.5 µL of sample with 1 µL of loading dye (Thermo Scientific, USA). Afterwards, the gel was observed under UV chamber for DNA bands (UV transilluminator, UVP, USA).

2.10. Sequencing analysis

The samples from PCR product that showed DNA bands after the gel electrophoresis was sent for sequencing analysis to Eurofins Genomics (Ebersberg, Germany). Bio Edit software was used for editing the sequence results obtained from the Eurofins Genomics. Later, the cut DNA sequence was used to analyze, compare and identify using the NCBI website. Bacterial identity was selected which showed highest value of percentage of similarity that is between 98 to 100% (Cole et al, 2014).

2.11 Lactic Acid Analysis

Lactic acid analysis was performed using an Enzytech D-/L- Lactic acid kit (Darmstadt, Germany). The samples were prepared according to the description proportioned by the manufacturer.

2.12 Statistical Analysis

SigmaPlot software was used to conduct the Statistical analysis. Statistical analysis was performed to determine the variation in the measurements obtained between the samples and storage time. The statistical value was presented as a median ± IQR. MannWhitney rank sum test was used to perform pairwise comparison between groups. Comparison was made using ANOVA on rank basis. The Pearson correlation coefficient (p) was used, and level of statistical significance was considered if $p<0.05$ value.

3. RESULTS

3.1. Formulation of quinoa milk

In the Pre-trials, varying amount of quinoa grains was used to determine the appropriate quantity required for preparing the quinoa milk. 50g of raw quinoa grains (white quinoa) was considered to be very little since they resulted in over toasting of the grains in lesser time, also the grains were un cooked, quantity of the quinoa milk obtained was too low and nutty smell was not observed. Hence, the proportion was modified. 15 minutes-soaked grains were observed to be the best method when compared to 1 hour and 12 hours of soaking. When the grains were soaked for 1 hour and 12 hours, it resulted in germination of the seeds which in turn produced off flavored milk and increased toasting time, most of them were stuck to the pan and some quickly got burnt. Besides, the quinoa grains were difficult to filter minimizing the product volume after filtration.

3.2. Measurement of pH

The decrease on the pH for each strain was measured before inoculation and after 48 hours of fermentation (after inoculation) and during storage that is on the 14^{th} day of storage and 28^{th} day of storage. Duration was chosen from the pre-trials conducted which was seen to be ideal period (from *Table* 2). From the results obtained, there is a decrease in pH for both the strains after fermentation. For *L. pentosus* 2 after 48 hours of fermentation, it was found a decrease below 4 (3.41). During storage time the pH further reduced to 3.37 at 14^{th} day. Thereafter there is slight increase in pH at 28^{th} day (3.43).

For *P. pentosaceous* 5, changes in pH were below 4 on the 14^{th} day (3.57) and the pH tends to remain constant on the 28^{th} day (3.58).

Table 2. *pH changes observed during quinoa milk fermentation.*

Sample	Before fermentation pH	48 hours Incubation pH	14 Days Storage time	28 Days Storage time
2	6.57	3.41	3.37	3.43
5	6.42	4.04	3.57	3.58

The analysis of variance presented a significant statistically analysis between 0 hour (before fermentation) and 48 hours (after fermentation) with P <0.05. However, there is no significant difference after 48 hours of fermentation and during storage (14 days and 28 days) which means that there is negligible difference indicating the values after 48 hours fermentation is relatively constant.

From *Figure 1*, A clear picture can be determined that pH changes after 48 hours of fermentation for both the strains. Thereafter, the pH tends to remain constant more or else for the entire storage period after 28 days.

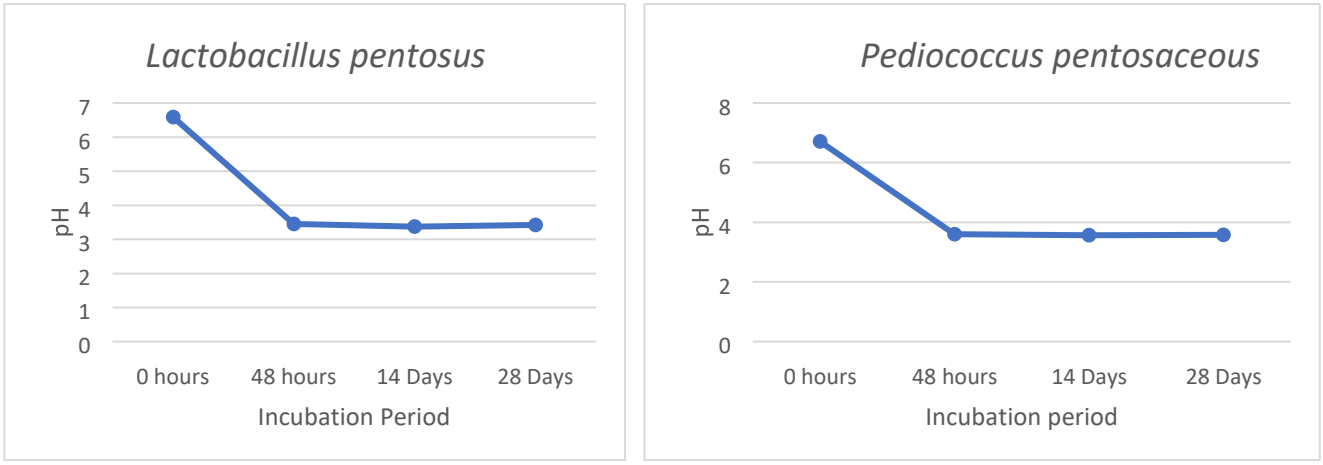

Figure 1: Changes of pH during fermentation and storage time.

3.3. Microbial count

To characterize the microbiota of the quinoa milk, samples were cultured on four different media. The number of viable cells from the fermented quinoa milk inoculated with *L. pentosus* 2 (Table 3; Figure 2)were found in high amount after fermentation recorded from Rogosa, TSA and MALT. However, before fermentation the number of viable *Enterobactericeae* VRBD was found to be 3.16 Log CFU/mL, and after fermentation no growth was observed. With respect to Lactobacilli, growth was not observed before fermentation and the highest amount was found to be 8.72 Log CFU/mL after 48 hours of fermentation and the bacterial count remained constant throughout the storage period up to 28 days. Before fermentation, bacterial count on

TSA and MALT is found to be 4.82 Log CFU/ml and 3.01 Log CFU/ml, highest value is found to be after 48 hours of fermentation with a value of 8.77 Log CFU/ml and 8.06 Log CFU/ml and the bacterial count was observed to be constant throughout the storage period.

Statistical analysis proves that there is statistically significant difference between 0 hours and 48 hours of fermentation and during storage for TSA, ROGOSA and MALT with a P value <0.05. However, for VRBD there is statistically significant difference between 0 hours and 48 hours of fermentation and after the fermentation process there is no significant difference (P>0.05) which makes sense since there is no growth of *Enterobactericeae* after fermentation process.

Table 3*. Viable count of fermented quinoa milk inoculated with L. pentosus 2.*

Incubation Period	**VRBD** (log CFU/ml)	**TSA** (log CFU/ml)	**ROGOSA** (log CFU/ml)	**MALT** (log CFU/ml)
0 hour (Before Inoculation)	3.16 ± 2.90	4.82 ± 0.07	0	3.01 ± 0.21
48 hours (After Fermentation)	0	8.77 ± 0.04	8.72 ± 0.07	8.73 ± 0.09
14 Days (Storage)	0	8.00 ± 0.27	7.96 ± 0.35	8.06 ± 0.31
28 Days (Storage)	0	7.48 ± 0.19	7.55 ± 0.12	6.24 ± 1.91

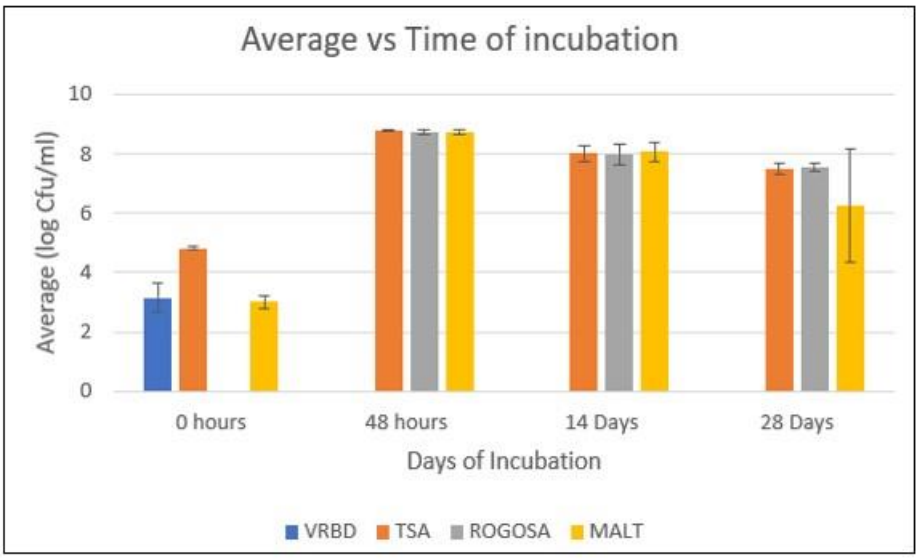

Figure 2. *Changes in microbial count during fermentation and storage after fermentation for te quinoa drink inoculated with L. pentosus 2*

The number of viable cells recorded from the fermented quinoa milk inoculated with *P. pentosaceous* also presented changes on the microbiota composition before and after fermentation (Table 4; Figure 3). Before fermentation the number of *Enterobactericeae* was 3.34 Log CFU/mL, and after fermentation no growth was observed. With respect to lactobacilli, growth of lactic acid bacteria was not observed before fermentation and growth was seen after the fermentation process with the highest values of 8.76 Log CFU/mL after 48 hours of fermentation and the bacterial count remained constant throughout the storage period up to 28 days. Similar results were observed on Malt extract agar, where the highest value was determined after 48 hours of fermentation with a value of 7.66 Log CFU/mL. Before fermentation, bacterial count on TSA was observed to be 4.93 Log CFU/mL, highest value is observed after 48 hours of fermentation with a value of 8.74 Log CFU/mL the bacterial count was observed to be relatively constant throughout the storage period which is until 28 days.

Statistical analysis proves that there is statistically significant difference between 0 hours and 48 hours of fermentation for TSA, ROGOSA and MALT with a p value <0.05. During storage, there is significant difference observed with p value <0.05. However, for VRBD there is statistically significant difference between 0 hours and 48 hours of fermentation and after the fermentation process there is no significant difference after fermentation process. There is no statistically significant difference ($p>0.05$) between 14^{th} day and 28^{th} day during storage on TSA.

Table 4 *Viable count of fermented quinoa milk inoculated with P. pentosaceous 5.*

Incubation Period	**VRBD** (log CFU/ml)	**TSA** (log CFU/ml)	**ROGOSA** (log CFU/ml)	**MALT** (log CFU/ml)
0 hour (Before Inoculation)	3.34 ± 2.69	4.93 ± 0.04	0	0
48 hours (After Fermentation)	0	8.74 ± 0.12	8.76 ± 0.09	7.66 ± 0.10
14 Days (Storage)	0	7.79 ± 0.17	7.81 ± 0.12	7.37 ± 0.09
28 Days (Storage)	0	7.66 ± 0.25	7.37 ± 0.17	7.54 ± 0.07

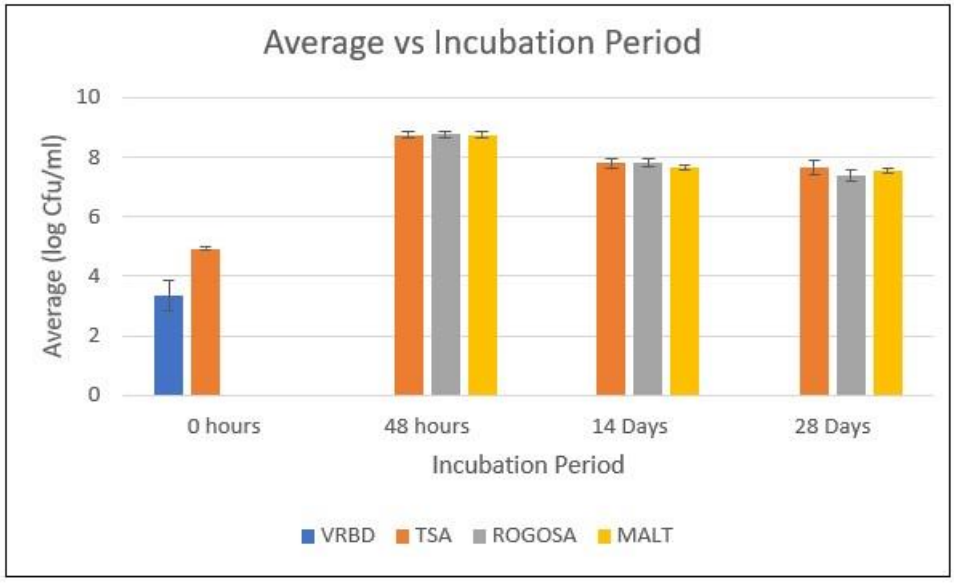

Figure 3*. Changes in microbial count during fermentation and storage after fermentation for the quinoa milk inoculated with P. pentosaceous 5.*

The survivability of the bacterium strains is expressed as percentage and represented the values during storage time after fermentation (Table 5). From the result obtained, survivability percentage of LAB is found to be 91.31% for *L. pentosus* 2, and 89.11% for *P. pentosaceous* on 14th day of storage. At the end of 28 days of storage, LAB percentage survivability is decreased in all the media. Statistical analysis shows that there is statistically significant difference during storage time for lactobacilli, total count, and mold or yeast (p value <0.05) for *L. pentosus* 2. However, for *P. pentosaceous* 5 there is a significant difference in lactobacilli and mold or yeast (p <0.05). There is no significant difference in total count (p>0.05).

Table 5. *Survivability percentage of bacteria in ROGOSA, TSA and MALT media*

Incubation Period	***L. pentosus* 2**			***P. pentosaceous* 5**		
	ROGOSA	**TSA**	**MALT**	**ROGOSA**	**TSA**	**MALT**
14 Days	91.31%	91.22%	92.28%	89.11%	89.09%	87.61%
28 Days	86.61%	85.29%	71.47%	84.15%	87.67%	86.25%

3.4 Concentration of Bacteria

Standard curve for determining the bacterial concentration of the starter culture was plotted *(Figure 4; Figure 5)*. The graph was used to for obtain the concentration of the starter cultures (*Table 6).*

***Table 6**. Concentration of starter cultures expressed as CFU/mL*

Sample No.	*L. pentosus* 2	*P. pentosaceous* 5
1	$8.3x10^8$	$1.1\ x10^9$
2	$7.7x10^8$	$9.8x10^8$
3	$7.3\ x10^8$	$1.2\ x10^9$
4	$1.2\ x10^9$	$1.2\ x10^9$

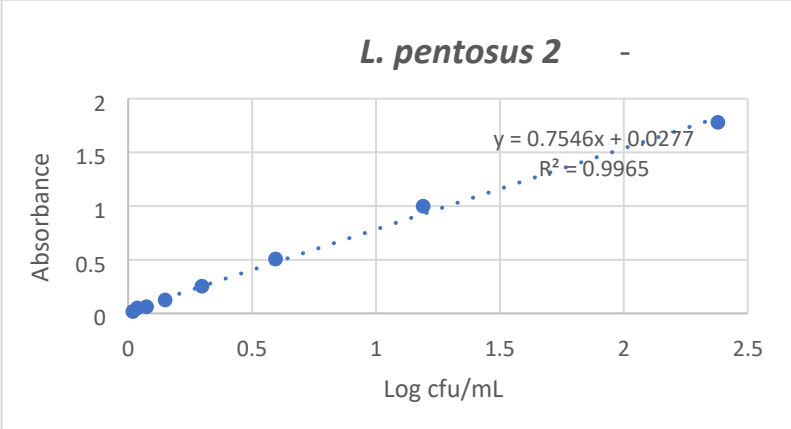

***Figure 4**. Standard curve for obtaining the L. pentosus 2 as starter culture concentration*

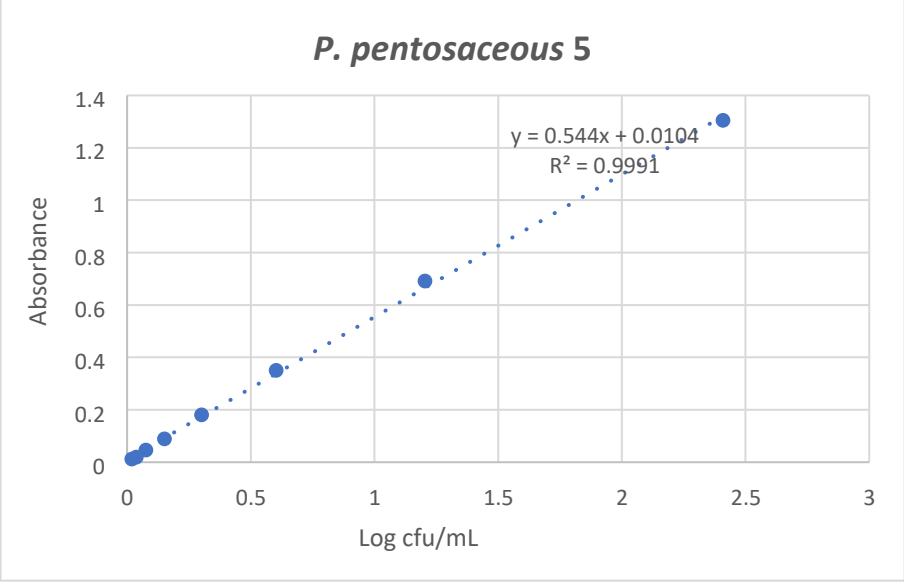

***Figure 5.** Standard curve for obtaining the P. pentosaceous 5 concentration*

3.5 Lactic Acid analysis

The concetration of D-/L- Lactic acid in the quinoa milk can be observed in the *Table 7*. From the results obtained, it is observed that the highest concentration of D- Lactic acid and L- lactic acid found was 7.70 g/L after 28 days of storage time, followed by 5.86 g/L after 14 days of storage for the quinoa milk inoculated with *L. pentosus* 2. Instead, for *P. pentosaceous* 5 the highest D- Lactic acid and L- lactic acid was 6.54 g/L and 4.48 g/L after 14 days of storage time. Statistical analysis shows that there is a statistically significant difference between 0 hour (before fermentation) and 48 hours (after fermentation) with $p < 0.05$. However, there is no significant difference after 48 hours of fermentation and during storage (14 days and 28 days) which means that there is negligible difference indicating the values after 48 hours fermentation is relatively constant.

Table 7. *Changes in lactic acid content before fermentation, after fermentation and during storage time expressed as g/L*

	***L. pentosus* 2**		***P. pentosaceous* 5**	
Incubation period	**L- Lactic**	**D- Lactic**	**L- Lactic**	**D- Lactic**
0 hours (Before Fermentation)	0.62 ± 0.57	0.46 ± 0.34	0.28 ± 0.47	1.17 ± 0.27
48 hours (After fermentation)	7.42 ± 0.75	5.09 ± 0.73	5.52 ± 2.79	3.02 ± 1.02
14 Days (During storage)	7.58 ± 1.03	5.86 ± 1.50	6.54 ± 1.07	4.48 ± 1.27
28 Days (During storage)	7.70 ± 1.09	2.97 ± 0.65	5.91 ± 0.91	3.16 ± 0.60

A correlation representing the relationship between pH and D-/L- Lactic acid content at the corresponding sampling times for the fermented quinoa milk is represented in Figure 6 and Figure 7. It can be seen that, as the fermentation process progresses, pH decreases while the D/L-lactic acid content increases.

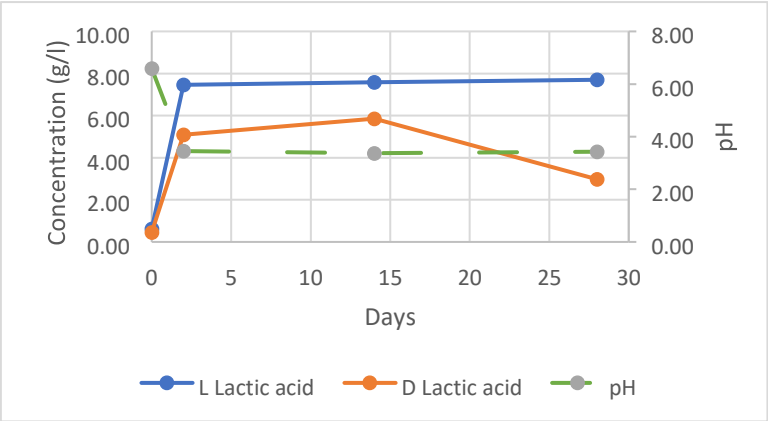

Figure 6: Comparison with changes in pH and lactic acid content during fermentation and storage for L. pentosus 2

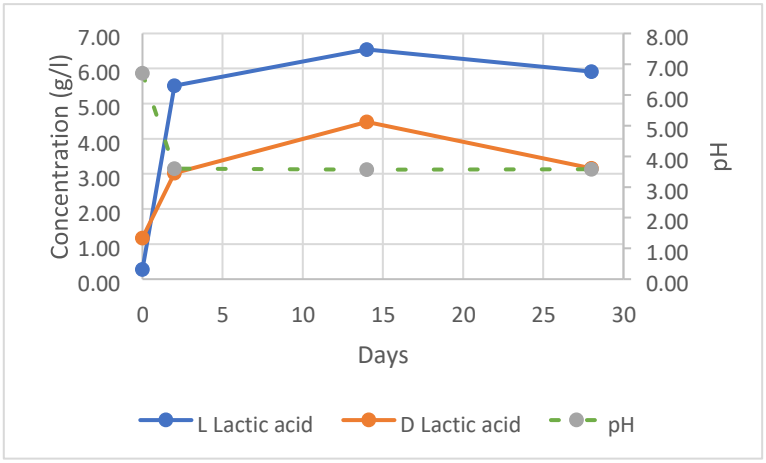

Figure 7: Comparison with changes in pH and lactic acid content during fermentation and storage for P. pentosaceous

3.6 RAPD

The bacteria isolated from Rogosa agar were used for RAPD analysis. RAPD analysis was carried out for all the incubation period that is 0 hours (Before fermentation), 48 hours (After fermentation), 14 Days and 28 Days. One example of RAPD analysis image for 48 hours and 14 Days from fermented quinoa milk is given in the *figure 8* and the other gel pictures are included in the Appendix E.

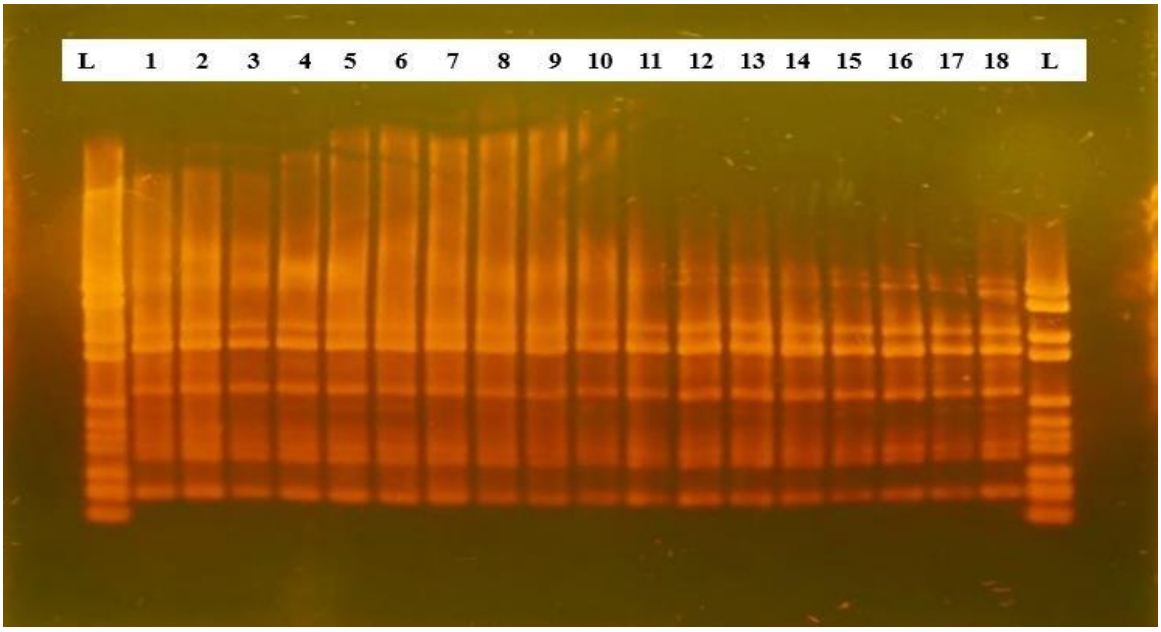

Figure 8: Agarose gel of amplified DNA extracted from bacteria found on fermented quinoa milk inoculated with L. pentosus 2. L stands for ladder and the numbers represent the RAPD samples

RAPD analysis image for 48 hours and 14 days from fermented quinoa milk inoculated with *P. pentosaceous* 5 is given in the *figure 9* and the other gel pictures are included in the Appendix F.

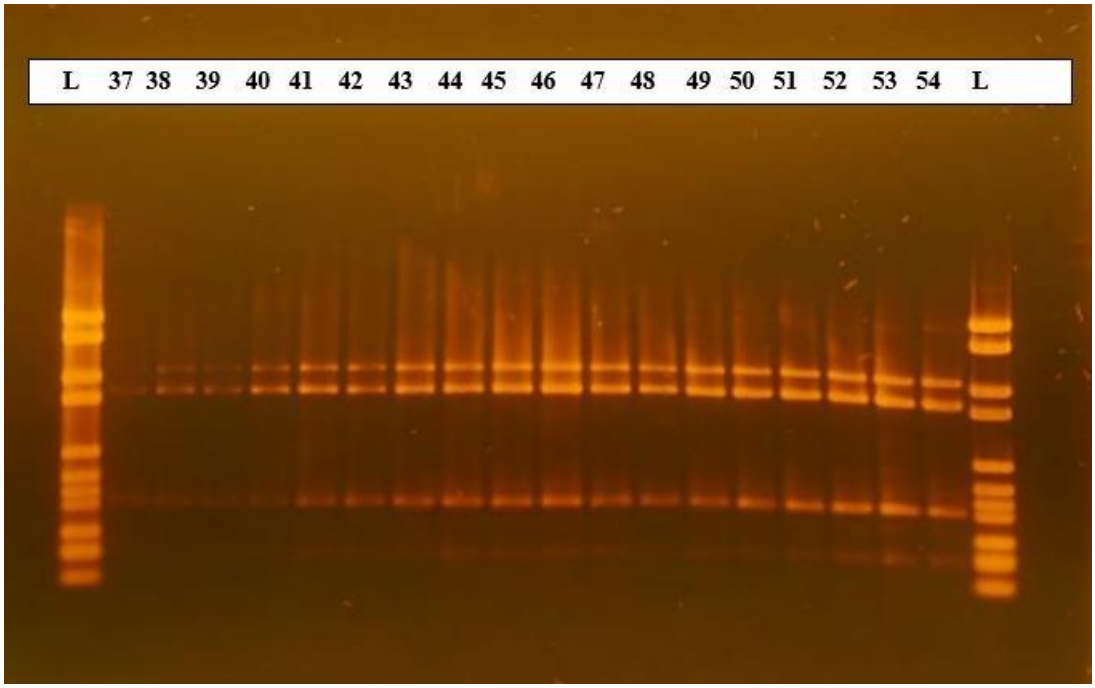

Figure 9: Agarose gel of amplified DNA extracted from bacteria found on fermented quinoa milk inoculated with P. pentosaceous 5 L stands for ladder and the numbers represent the RAPD samples

3.7 Identification of bacteria from fermented quinoa milk

Identified names of the bacterial species and its percentage of similarity are listed on Table 8 and Table 9 for the viable cells isolated from the fermented quinoa milk using as starter culture *L. pentosus* 2, and Table 10 and Table 11 for the starter culture *P. pentosaceous* 5.

Table 8. *Sequencing results from the inoculated quinoa milk isolates cultivated in Rogosa and TSA media.*

Incubation Period	**ROGOSA media**		**TSA media**	
	Identified bacteria & % of identity		**Identified bacteria & % of identity**	
0 hours (Before Fermentation)			*Klebsiella michiganensis* *Paenibacillus tundrae* *Chryseobacterium oranimense*	100 99.8 99.7
48 hours (After Fermentation)	*Lactobacillus paraplantarum* *Lactobacillus pentosus* *Lactobacillus plantarum*	100 99.9 99.9	*Lactobacillus paraplantarum* *Lactobacillus pentosus* *Lactobacillus plantarum*	100 99.9 99.9
14 days (Storage)	*Lactobacillus paraplantarum* *Lactobacillus pentosus*	100 99.9	*Lactobacillus paraplantarum* *Lactobacillus pentosus* *Lactobacillus plantarum* *Kocuria marina*	100 99.9 99.9 99.6
28 days (Storage)	*Lactobacillus paraplantarum* *Lactobacillus pentosus* *Lactobacillus plantarum* *Lactobacillus xiangfangensis*	100 99.9 99.9 98.2	*Lactobacillus paraplantarum* *Lactobacillus pentosus*	100 99.9

Table 9. *Sequencing results from the isolates cultivated in MALT and VRBD media.*

Incubation Period	**MALT media**		**VRBD media**	
	Identified bacteria & % of identity		**Identified bacteria & % of identity**	
0 hours (Before Fermentation)	*Klebsiella michiganensis* *Leclercia adecarboxylata* *Leclercia adecarboxylate*	100 100 100	*Klebsiella michiganensis* *Leclercia adecarboxylata* *Leclercia adecarboxylate*	100 99.6 99.6
48 hours (After Fermentation)	*Lactobacillus pentosus* *Lactobacillus plantarum* *Lactobacillus paraplantarum*	100 100 100		
14 days (Storage)	*Lactobacillus paraplantarum* *Lactobacillus pentosus* *Lactobacillus plantarum*	100 99.9 99.9		
28 days (Storage)	*Lactobacillus pentosus* *Lactobacillus plantarum*	100 100		

***Table 10**. Sequencing results from the isolates cultivated in Rogosa and TSA media.*

Incubation Period	ROGOSA media		TSA media	
	Identified bacteria & % of identity		**Identified bacteria & % of identity**	
0 hours (Before Fermentation)			*Klebsiella michiganensis* *Leclercia adecarboxylata* *Micrococcus terreus*	100 99.6 99.1
48 hours (After Fermentation)	*Lactobacillus paraplantarum* *Lactobacillus pentosus* *Lactobacillus plantarum*	100 100 99.9	*Lactobacillus paraplantarum* *Lactobacillus pentosus* *Lactobacillus plantarum* *Kocuria marina* *Kocuria indica*	100 99.9 99.9 99.8 99.2
14 days (Storage)	*Lactobacillus paraplantarum* *Lactobacillus pentosus* *Lactobacillus plantarum*	100 99.9 99.9	*Lactobacillus paraplantarum* *Lactobacillus pentosus* *Lactobacillus plantarum*	100 99.9 99.9
28 days (Storage)	*Lactobacillus paraplantarum* *Lactobacillus pentosus* *Lactobacillus plantarum*	99.9 99.8 99.8	*Lactobacillus paraplantarum* *Lactobacillus pentosus* *Lactobacillus plantarum*	100 99.9 99.9

***Table 11.** Sequencing results from the isolates cultivated in MALT and VRBD media.*

Incubation Period	MALT media		VRBD media	
	Identified bacteria & % of identity		**Identified bacteria & % of identity**	
0 hours (Before Fermentation)			*Pseudomonas koreensis* *Pseudomonas moraviensis* *Klebsiella michiganensis* *Leclercia adecarboxylata* *Stenotrophomonas maltophilia* *Stenotrophomonas pavanii* *Pseudomonas asplenii* *Pseudomonas fuscovaginae*	100 100 100 99.6 99.9 99.7 99.3 99.3
48 hours (After Fermentation)	*Lactobacillus pentosus* *Lactobacillus plantarum* *Lactobacillus paraplantarum*	99.7 99.7 99.9		
14 days (Storage)	*Lactobacillus pentosus* *Lactobacillus plantarum*	99.9 99.9		
28 days (Storage)	*Lactobacillus pentosus* *Lactobacillus plantarum*	100 100		

4. DISCUSSION

The pre-treatment on which the grains were submitted increased the acceptability of the product. In both the case, nutty smell and less bitter flavour in comparison to the distinct vegetable smell of the unfermented quinoa milk.

Furthermore, it was obtained the fermented quinoa milk using *Lactobacillus pentosus* and *Pediococcus pentosaceus* as stater cultures. All the fermented quinoa milk with *L. pentosus* and *P. pentosaceus* reached pH below 4 after 48 hours of incubation at 30°C. Which was determined by monitoring the fermented quinoa milk. These findings help to determine the ideal time required for fermentation process to be effective on the quinoa milk. A study by Barba *et al* also reported similar results for *Lactobacillus pentosus* where the pH reduced significantly within 48 hours (Barba *et al*, 2012). According to the literature, *P. pentosaceus* is said to grow better at temperature around 40-45°C (Molin, 2013), this study showed that *P. pentosaceus* was able to ferment effectively at 30°C as well.

Analyses of the result is done in two sections first being before and after fermentation and second includes during storage which is at 14 days and 28 days. Before fermentation process, pH of the quinoa milk was found to be 6.59 and 6.71. After 48 hours of incubation, pH of *L. pentosus* (2) and *P. pentosaceus* (5) reached 3.41 and 3.59. Te pH values remained stable during storage time, which was statistically demonstrated. The findings were also observed by other studies such as the decrease in pH from 0 to 48 hours is also found in similar study with fermented quinoa beverage (Bianchi et al, 2015).

In this study, *Lactobacillus plantarum*, *Lactobacillus pentosus* and *Lactobacillus paraplantarum* were identified from the fermented quinoa milk by 16S rRNA sequencing however the *Lactobacillus pentosus* was used as a starter culture. *L. plantarum* is said to be one of the dominating lactic acid bacteria found in the fermented food especially when the foods are plant based (Molin, 2013). *L. plantarum* and *L. pentosus* are said to be genotypically related species (Molin, 2013) and possess high phenotypes (Torriani et al, 2001). Studies shows that 16S rDNA sequence for *L. plantarum* and *L. pentosus* are similar and yet difficult to differentiate because of high identity percentage of 99% (Torriani et al, 2001). This could be one of the possible reason for obtaining *L. plantarum* and *L. pentosus* as the identified sequence for the fermented quinoa milk. In case of the quinoa milk using *P. pentosaceous* as starter

culture, *Lactobacillus plantarum*, *Lactobacillus pentosus* were identified even though the starter culture was *Pediococcus pentosaceus*. One of the possible reasons could be that *L. plantarum* is one of the most dominating bacteria that is found in the fermented plant-based food that suppressed the growth of *P. pentosaceus* in the quinoa milk.

Additionally, the fermented quinoa milk content more than 10^6 CFU/ml live lactic acid bacteria in the final fermented product as well as during the storage for up to 28 days. As mentioned earlier in the background, any food product containing more than 10^6 CFU/ml live probiotic bacteria is considered as a probiotic food. Therefore, the fermented quinoa milk can be considered as a probiotic drink.

As the pH of the fermented milk reduces there is significant increase in the lactic acid content. Similar results were observed in another study dealing with use of lactic acid bacteria developing novel beverage made of quinoa flour (Lorusso et al, 2018).

During storage period, result on 14^{th} day and 28^{th} day showed increased amount of lactic acid content which means that there is continued production of lactic acid which exhibits the activity of the lactic acid bacteria in the fermented quinoa milk.

5. CONCLUSIONS

The aim of this study was to analyze the ability of potential lactic acid bacteria to ferment quinoa milk as the main objective. This was achieved by formulating the method for quinoa milk preparation. Optimization of the method was obtaining by considering the amount of quinoa grains, temperature, time, mixing rate and filtration method. Colour, taste and smell were considered for final optimized method. The results from this study confirms that *Lactobacillus pentosus* and *Pediococcus pentosaceus* isolated from white quinoa grains has the ability to ferment quinoa milk. There was a statistically significant decrease in pH and increase in lactic acid content after 48 hours of fermentation. At storage, there was no significant difference in the pH and lactic acid content which means that pH and lactic acid after fermentation remained relatively constant. Additionally, fermented quinoa milk with both the bacterial strains contained bacteria between 2.6×10^7 CFU/ml to 5.9×10^8 CFU/ml for *P. pentosaceus* and 4.2×10^7 CFU/ml to 3.8×10^8 CFU/ml for *L. pentosus*, there the fermented quinoa milk can be considered as a probiotic drink.

It can be concluded that, *L. pentosus* isolated from white quinoa possess better ability to ferment quinoa milk compared to *P. pentosaceus* in this study. With regard to pH, acidity as well as survivability during storage in *L. pentosus* shows better results. Furthermore, this study gives way for future research. In the future, it can be replicated with the addition of prebiotics in order to enhance the fermentation process, also enhancing the sensory properties of the milk. Evaluating the ability of other strains of lactic acid bacteria to ferment quinoa milk and sensory evaluation of the fermented quinoa milk can be considered for future experiments.

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APPENDICES

Appendix A:

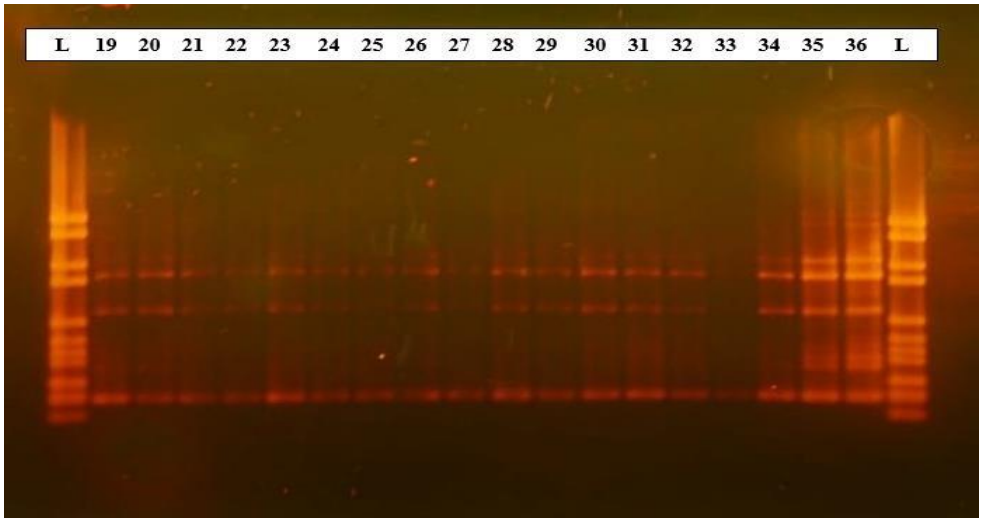

Figure 1: *Agarose gel of amplified DNA extracted from bacteria found on fermented quinoa milk inoculated with 2 from ROGOSA plate. L stands for ladder and the numbers represent the RAPD samples*

Appendix B:

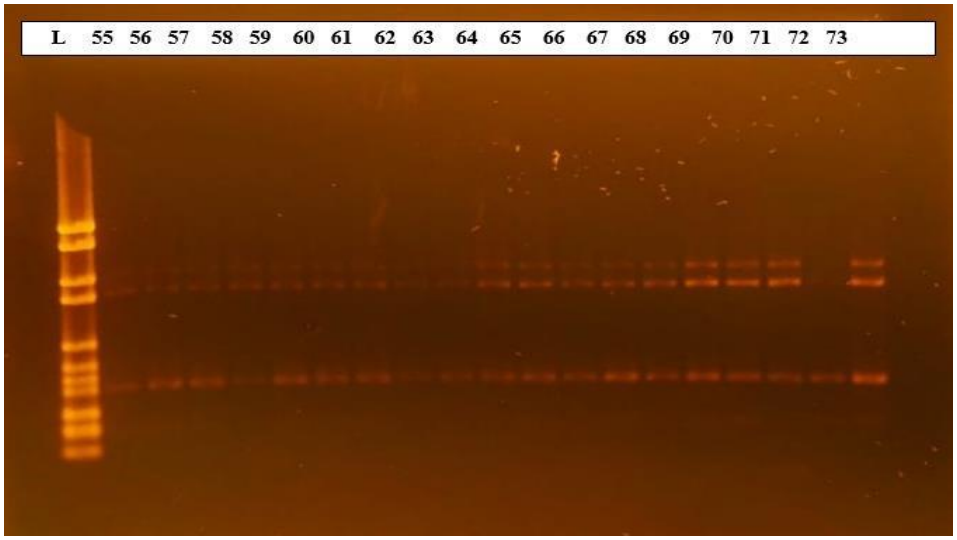

Figure 2: *Agarose gel of amplified DNA extracted from bacteria found on fermented quinoa milk inoculated with 5 from ROGOSA plate. L stands for ladder and the numbers represent the RAPD samples*