Enhancement of Probiotic Yogurts' Characteristics Using a 24-hour Fermentation Process

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ABSTRACT

Probiotic yogurts are dairy products that contain beneficial live microorganisms that remain in sufficiently high numbers after being exposed to stomach acidity. Baladna, Al Maha and Rawa are local yogurts that have been commercialized in Qatar after the economic blockade was imposed on it in 2017, and their labelling does not indicate the species they include nor their CFU count per ml. This study is not only the first that attempts to characterize these yogurts as compared to imported probiotic yogurts (Yeo and Activia), but also tests a protocol aimed at enhancing some of their characteristics, including resistance to acidity, probiotic content, antibiotic property against pathogens and antibiotic resistance. The commercial yogurts available locally were incubated with local cow milk for 24 hours at 37 °C. Baladna and Rawa, showed significantly better resistance to acidity than Activia and/or Yeo probiotic yogurts and contain enough probiotic bacteria to be considered as probiotics. The yogurt BB37/24, which used Baladna as starter, showed the best results overall with the highest resistance to acidity than all yogurts including Activia, the highest abundance of *Streptococcus thermophilus* of all yogurts, the highest antibiotic property against *Pseudomonas aeruginosa* among lab synthetized yogurts, and the least resistance to streptomycin, chloramphenicol and tetracyclin. This study supports the benefits of using a longer fermentation during the production of probiotic yogurts.

Introduction

Fermentation of dairy and non-dairy products is an ancestral culinary process of many cultures. However, it was only recently that the benefits of the microbes contained in those fermented products have undergone serious scientific study. Because evidence of the beneficial role played by the gut microbiota in health and disease is growing [1], [2], identification of the probiotic strains and characterization of these products is becoming essential. Probiotics are "live microorganisms which, when administered in adequate amounts, confer a health benefit on the host" (World Health Organization : Food and Agriculture Organization of the United Nations, 2006). An imbalanced gut microbial population has been associated with diseases and disorders in the host metabolism; these include inflammatory bowel diseases (IBD), cancer, cardiovascular diseases, and metabolic syndrome [4]. While the safety of fecal transplants to reestablish eubiosis is being investigated across many clinical trials worldwide, there is robust evidence for the use of probiotics as a solution for amending microbial imbalances and as a necessary adjunct to antibiotic treatments that could lead to dysbiosis of human gut microbiota [5]. Probiotics have also proven beneficial for disease prognosis and management. Furthermore, specific bacterial strains have shown to be effective in the treatment of various conditions: antibiotic-associated diarrhea [6]; prevention of *C. difficile*-associated diarrhea [7], [8] IBD [9]–[12]; Autism and neurologic diseases [13]–[15]; mental health [16], [17].

The WHO has established the conditions required for a product to be considered as a "probiotic": 1/ identified strains; 2/ minimum number of 2 billion colony forming units (CFU); 3/ resistance to acidity/bile acids; 4/ Antibiotic properties against pathogens or other benefits to host.

Following these recommendations, we've designed the study to enhance and characterize three local yogurts – Baladna, Al Maha, Rawa – and compare them to yogurts which already claim as being probiotics such as Activia and Yeo. The present publication describes the preliminary results in term of: survival of microbial communities to stomach acidity, type and abundance of lactic acid bacteria, antibacterial property against pathogens and antibiotic resistance. Because studies have pointed to an emergence, in Qatar, of multidrug resistant strains of *Serratia marcesens* [18] and *Pseudomonas aeruginosa* [19], we chose these two pathogens for preliminary testing of these yogurts' antibacterial properties. As for the resistance to antibiotics, we chose four antibiotics commonly used in healthcare, with the reasonning resistance to antibiotics need to be maintained in order to avoid the spread of resistance genes to pathogenic microbes via conjugation and preventing the introduction of more resistance genes into the host. Indeed, development of resistant microbes is mainly due to the presence of resistance genes and the selective pressure resulting from the use of antibiotics [20]. Therefore, the antibiotics chosen are: 1: chloramphenicol, because it is used against a variety of bacterial infections [21]; 2: tetracylin, employed for the treatment of infections relating to skin bacteria, the intestine, respiratory tract, urinary tract and genitals [22]; 3: Streptomycin, used for the treatment of severe infections such as tuberculosis, pneumonia and E.coli [23]; 4: Penicillin, used against severe infections, such as, meningitis, anthrax, and pneumonia [23].

LAB can be divided into two groups based on their optimum temperature of growth: mesophilic (20 to 30°C) and thermophilic (30 to 45°C). Work by Yung and colleagues [24], was conducted to evaluate the quality of the fermented yogurt at different temperatures. Fresh milk was used as a raw material and fermentation strains such as *Lactobacillus bulgaricus* and *Streptococcus thermophilus* were used to yield yogurt at different fermentation temperatures (30, 37, 40, 42, and 45 °C). The study found that the highest content of essential amino acid was at 37 °C and that fermentation at 37 °C was better in improving its nutritional qualities including essential fatty acids such as linoleic and linolenic acids. Horiuchi and colleagues found that fermentation at 37°C resulted in a product with less hardness and a smoother texture [25]. However, at low-temperature fermentation, it would reduce the yogurt manufacturing efficiency as the yogurt takes longer to process. Therefore, and after some preliminary testing, we have chosen to perform the fermentation at 37°C.

Prior to the economic blockade imposed on it in 2017, Qatar was heavily reliant on exported dairy products such as Activia or Greek yogurts for their probiotic benefits. Activia states its probiotic content in its labelling and website, stating that it includes *Lactobacillus bulgaricus, Streptococcus thermophilus, Lactococcus lactis* [26]. The Yeo brand also states the probiotic bacteria in its labelling and list *Bifidobacterium, Lactobacillus Bulgaricus, Streptococcus Thermophilus* as its contents. *(Yeo Valley Production).*

Since 2017, Qatar has developed numerous dairy brands, including the brands Baladna, Rawa and Al Maha. All the local brands still lack adequate data on whether the yogurts contain enough of these strains to be considered as probiotics. The identification of bacteria contained in these yogurts are also not present in their labeling. The yogurts we have chosen to use as starters are therefore: Baladna, Al Maha, Rawa, Yeo and Activia, which respectively yielded after intervention the BB37/24, MB37/24, RB37/24, GB37/24 and AB37/24.

One of the main parameters that drives choice of consuming dairy or non-dairy probiotic is lactose intolerance [28]. Indeed, the insufficient resorption of lactose in the small intestine (caused by a reduced activity or the absence of β-galactosidase), induces gastrointestinal pain that discourages people from consuming dairy probiotics [29]. However, longer fermentation has been shown to reduce the amount of lactose present in the product [30]. Recent studies have suggested that the fermentation of dairy products that contain lactic acid bacteria (LAB), can reduce lactose intolerance symptoms for those affected [29]. Different combinations of LAB are being used by various brands of yogurts: *Lactobacillus casei* strain *Shirota (*Yakult*)*, *Lactobacillus rhamnosus*, *Lactobacillus paracasei* and *Propionibacterium freudenreichii subsp.shermanii* (Danisco), *Limosilactobacillus reuteri*, (BioGaia).

LAB have preservative properties and have the ability to ferment lactose into lactic acid [31]. A study conducted on kefir (a fermented yogurt drink) in the United States, utilized the method of incubating the pasteurized milk with bulk lyophilized kefir grain cultures for an optimal time period from 18 to 24 hours. The results showed that the brand of Kefir under study has the potential to be an alternative yogurt for lactose digestion improvement. The study concludes that more work is needed to explore the lactose digestion mechanism; more specifically there has been a call for comparisons of different starter cultural microorganisms and their impact on the digestion of lactose [32]. Although there is evidence that longer fermentation can reduce lactose content in yogurts and reduce lactose intolerance, there are little data on how resistance to acidity, probiotic abundance, antibiotic properties, and susceptibility to antibiotics do change after longer fermentation.

Therefore, this study aims to investigate the effect of a 24-hours fermentation at 37 C, by characterizing the original yogurts used as starters before the intervention, and the yogurts produced after implementation of this protocol.

Methods

Intervention Protocol and Experimental Design

Milk from the local brand Baladna was boiled and allowed to cool to 37°C. Six grams of yogurt starter (Baladna or Al Maha or Rawa or Activia or Yeo) was added to a 45 ml tube of warm milk. Tubes were mixed using a vortex for 3 seconds then incubated in aerobic conditions for 24 hours at 37°C. Yogurts were made in triplicates. The yogurts were labelled as per the starter, the milk used (baladna milk), followed by fermentation temperature and duration of fermentation in hours: BB37/24 when using baladna as starter, MB37/24 when using Al Maha, RB37/24 when using Rawa, AB/7/24 when using Activia, GB37/24 when using Greek yogurt Yeo. Figure 1 summarizes the experimental design and tests performed.

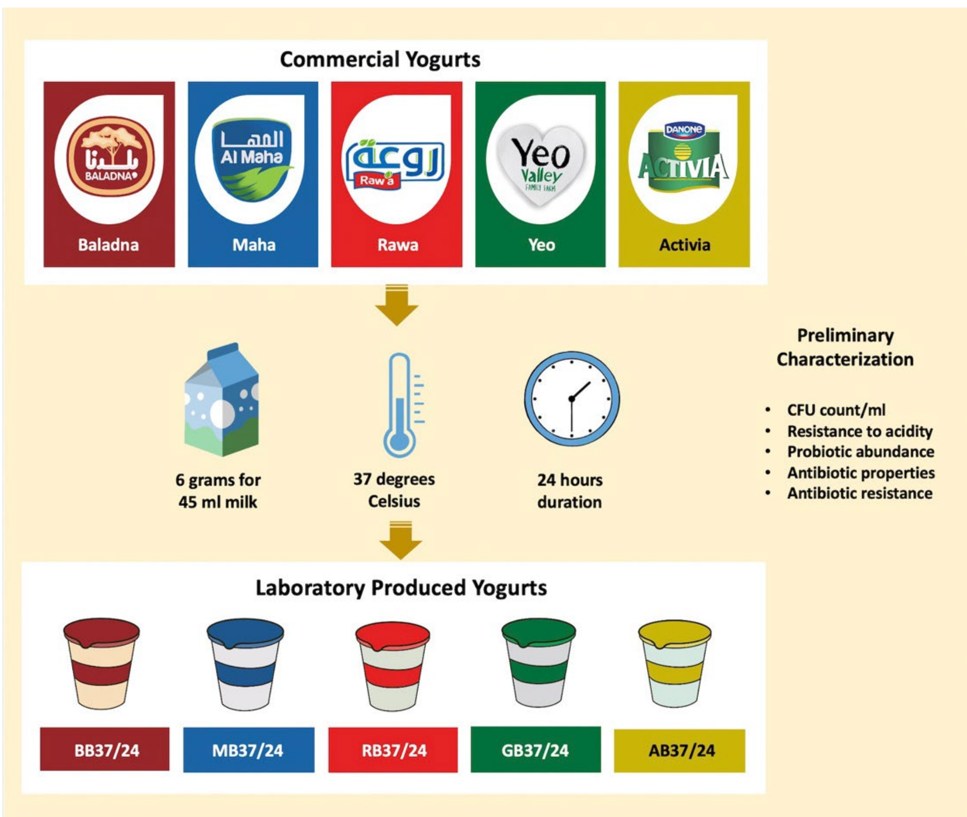

Figure 1. Experimental design.

Determination of the CFU/mL Count

A serial dilution was performed to 1/1,000,000 using M17 broth and 200 µL of dilution were plated in M17 agar plates. Plates were allowed to incubate in anaerobic conditions overnight at 37°C. Colony counting was done in the diluted plate and the CFU/ml count in the yogurt was concluded by multiplying the colony number by the dilution factor. The mean number between triplicates was considered for analysis.

Resistance to Acidity

A 1:10 dilution (one gram of yogurt added to 9mL of PBS (pH 2.5)) was performed before placing the tube in a shaking water bath for three hours at 37°C. The remaining serial dilutions were performed to 1/1,000,000 using M17 broth and 200 µL of the final dilution were plated in M17 agar plates. Plates were allowed to incubate in anaerobic conditions overnight at 37°C. Colony counting was done in the diluted plate and the CFU/ml count in the yogurt was concluded by multiplying the colony number by the dilution factor.

Testing Antibiotic Property

A volume of 0.1 µL of inoculum was added to 9.9 ml of M17 broth to grow overnight each of the pathogenic bacteria *Pseudomonas aeruginosa* or *Serratia marcesens*. A volume of 250 µL of the culture was plated in M17 agar plates. A pipet of a diameter of 8 mm was used to create a hole at the center of each agar plate, the volume of 200mm^3 was filled with yogurt (0.2 g) using a sterile spatula. Plates were incubated overnight in aerobic conditions and inhibition zones were measured using a ruler.

Antibiotic Resistance

The antibiotic resistance was tested using the disc diffusion method using disc loaded with P10 (penicillin), S10 (streptomycin), T30 (tetracycline), and C30 (chloramphenicol). For each yogurt, a suspension was grown overnight in M17 broth. A volume of 200 µL of the bacterial suspensions of yogurts were plated on M17 agar plates and incubated in aerobic conditions overnight. Inhibition zones were measured using a ruler.

DNA Extraction

The DNeasy Powerfood Microbial kit (Qiagen, Hilden, Germany) was used to extract DNA from the yogurts. Samples were homogeneized using a micropipette. A volume of 1.8ml of yogurt was added to a collection tube and processed using PowerBeads as per manufacturers' instructions. A volume 50 µL of elution buffer (instead of 100 µL) was used to elute the cleaned DNA. Samples were frozen at -20°C until analysis.

16sRNA Sequencing and Phylogenetic Analysis

Determination of abundance of probiotic bacteria in Qatari products were performed using 16sRNA sequencing and phylogenetic analysis were performed to determine the abundance of probiotic bacteria in Qatari products. The amplified DNA samples were all transferred to CD genomics (Shirley, NY, USA). The 16sRNA sequencing and phylogenetic analyses were carried out on the PE300 Illumina Miseq v3 sequencing platform with a 600-cyle format. Operational taxonomic unit (OTU) clustering, alpha-diversity analysis, OTU analysis/species annotation (phylogenetic tree, Venn graph, Heatmap, and taxonomic tree), and beta-diversity analysis (PCA analysis, PCoA analysis, unweighted UniFrac distance heatmap, UPGMA, and NMDS analysis) were all performed. CD genomics delivered a comprehensive report as well as raw data Excel files.

Statistical Analysis

Parametric *t*-testing was performed using Excel. Statistical significance was defined as a $p < 0.05$, *p*-value more than 0.05 were defined as non-significant (NS). All error values reported are standard deviations of the mean.

Results

Resistance to Acidity

Before Intervention

Commercial yogurts' resistance to acidity was tested and compared. The commercial yogurt Rawa showed better resistance to acidity than all other commercial yogurts (Table 1). Similarly, the Greek yogurt Yeo showed the second-best resistance to acidity among the commercial yogurts, with a significantly higher CFU count per ml than Baladna, Maha and Activia (Figure 2, Table 1). After the acidity test, the Baladna yogurt showed a significantly lower CFU count per ml than Al Maha, Rawa, Yeo. However, it was significantly higher than Activia, which showed the least resistant to acidity of the investigated commercial yogurts (Figure 2, Table 1).

Table 1. T-test p-values for the CFU/ml count after acidity test.

CFU/ml Count after acidity										
T-test p-values	BB37/24	MB37/24	RB37/24	GB37/24	AB37/24	Baladna	Al Maha	Rawa	Greek Yeo	Activia
BB37/24	x	x	x	x	x	x	x	x	x	x
MB37/24	0.000	x	x	x	x	x	x	x	x	x
RB37/24	0.000	0.270	x	x	x	x	x	x	x	x
GB37/24	0.000	0.075	0.354	x	x	x	x	x	x	x
AB37/24	0.000	0.252	0.655	0.852	x	x	x	x	x	x
Baladna	0.000	0.867	0.214	0.063	0.293	x	x	x	x	x
Al Maha	0.000	0.785	0.353	0.097	0.384	0.003	x	x	x	x
Rawa	0.001	0.004	0.070	0.010	0.053	0.000	0.000	x	x	x
Greek Yeo	0.000	0.019	0.052	0.117	0.297	0.000	0.000	0.000	x	x
Activia	0.000	0.655	0.090	0.030	0.177	0.000	0.000	0.000	0.000	x

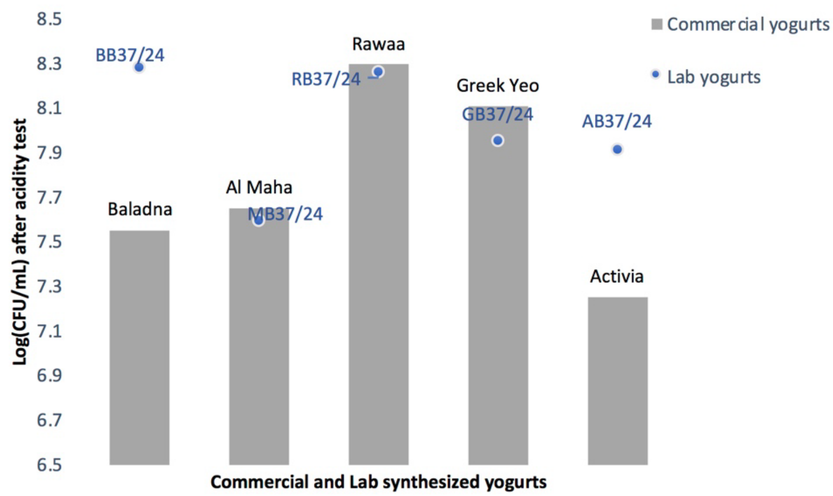

Figure 2. Comparing CFU/mL of all yogurts after the acidity test.

After Intervention

While the Baladna yogurt showed low CFU/ml, the applied protocol to make the developed BB37/24 resulted in a yogurt with significantly higher number of colonies forming units per ml than all other yogurts including the commercial yogurts that are recognized as probiotics (Figure 2, Table 1). This suggests that the protocol was successful in improving resistance to acidity of this yogurt. When this protocol was applied to Activia, it resulted in an AB37/24 with an increase of the CFU count per ml not significantly different from that found in the Yeo yogurt, suggesting an improvement of resistance to acidity. However, the standard deviation was large and the increase was not significantly different from Activia ($p=0.177$). The protocol did not significantly change resistance to acidity of Rawa yogurt in RB37/24, nor Maha yogurt in MB37/24, or the Yeo yogurt in GB37/24. The highest resistance to acidity was observed with the produced yogurt BB37/24, made out of Baladna yogurt, which was interestingly the least resistant before the applied protocol. BB37/24 shows significantly higher CFU count per ml than all other yogurts and commercial yogurts tested. RB37/24 shows significantly higher resistance to acidity than the Yeo. The GB37/24 show a significantly higher CFU count per ml than Activia. The yogurts produced out of Al Maha or Activia, respectively MB37/24 and AB37/24 are not significantly different. (Figure 2, Table 1).

Probiotic Abundance

The four most abundant bacteria found after 16sRNA sequencing were: *Streptococcus thermophilus, Lactobacillus Bulgaricus, Lactococcus Lactis, Bifidobacterium animalis*. Table 2 shows the absolute abundance of these four bacterial species in each of the yogurt tested after DNA extraction and 16sRNA sequencing.

Table 2. Absolute abundances of lactic acid bacteria in the yogurts tested.

Taxonomy	Baladna	Maha	Rawa	Yeo	Activia	BB37/24	MB37/24	RB37/24	GB37/24	AB37/24
Streptococcus thermophilus	1085	21379	16403	3097	363	7203	4013	2320	3430	3660
Lactococcus lactis	35	1770	62	20	79	18	2	454	0	14
Bifidobacterium animalis	32	148	110	99	79	24	87	20	188	247
Lactobacillus bulgaricus	22	6	39	81	3905	2	73	99	119	465

Before intervention (Figure 3), the most abundant species in Baladna, maha, Rawa and Yeo was *Streptococcus* thermophilus. However, in Activia, it was *Lactobacillus* bulgaricus. Al Maha yogurt has the highest amount of *Streptococcus* thermophilus, *Lactococcus* lactis and *Bifidobacterium animalis*. Rawa yogurt showed the second-highest amount of *Streptococcus thermophilus* and Activia has the least amount of *Streptococcus thermophilus*.

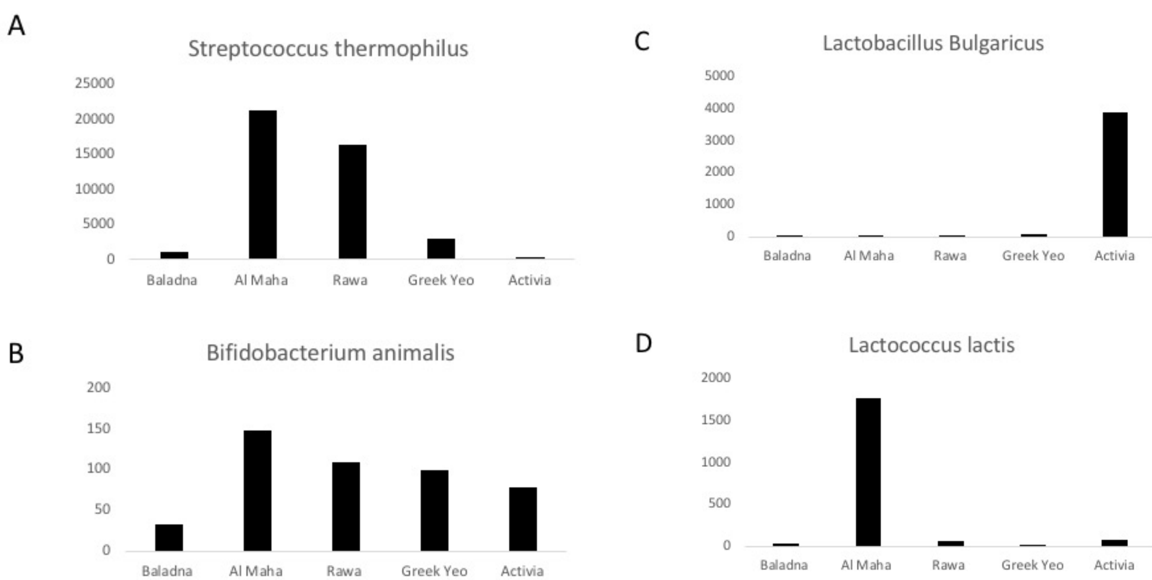

Figure 3. Absolute abundance of probiotic bacteria in commercial yogurts. Abundances of (A) *Streptococcus Thermophilus*, (B) *Bifidobacterium Animalis*, (C) *Lactobacillus Bulgaricus*, (D) *Lactococcus Lactis*.

After intervention (Figure 4), the abundance of *Streptococcus* thermophilus increased in BB37/24, GB37/24 and AB37/24, as compared to their starters respectively Baladna, Yeo and Activia. However, it decreased in Al Maha and Rawa. The abundance of *Lactococcus* lactis only increased in RB37/24 and decreased in other yogurts. The abundance of *Bifidobacterium* animalis and *Lactobacillus* bulgaricus increased in MB37/24, RB37/24, AB37/24 and decreased in other yogurts. The intervention seemed to induce shifts in the abundance of certain species.

When comparing all yogurts, the produced yogurts RB37/24 and AB37/24 made from Rawa and Activia respectively, showed the best equilibrium of the four species.

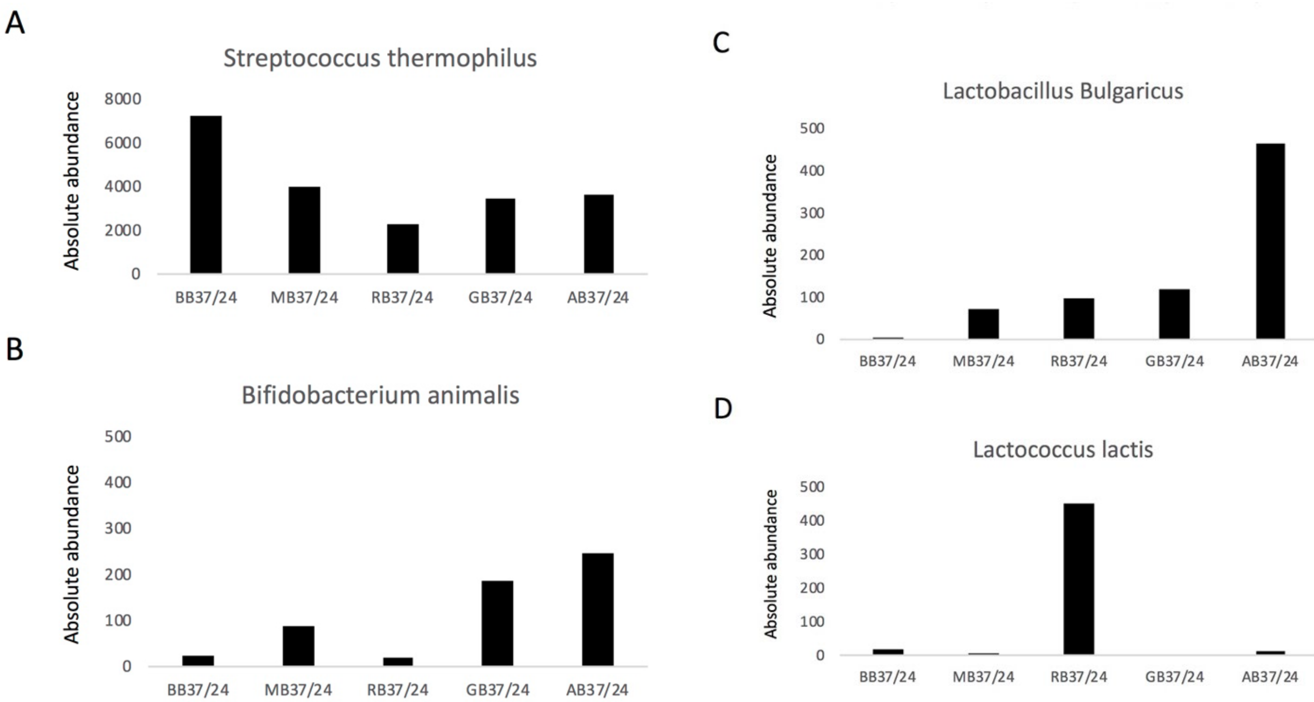

Figure 4. Absolute abundance of probiotic bacteria in lab synthetized yogurts. Abundances of (A) *Streptococcus Thermophilus*, (B) *Bifidobacterium Animalis*, (C) *Lactobacillus Bulgaricus*, (D) *Lactococcus Lactis*.

Antibiotic Properties

Antibiotic property of each of these yogurts was tested against *Pseudomonas aeruginosa* and *Serratia marscesens unsing the disc diffusion method* to test their capacity to inhibit their growth. The Greek yogurt Yeo induced significantly higher inhibition zones on *Pseudomonas aeruginosa than all other yogurts.* Activia showed similar activity (Figure 5, Table 3).

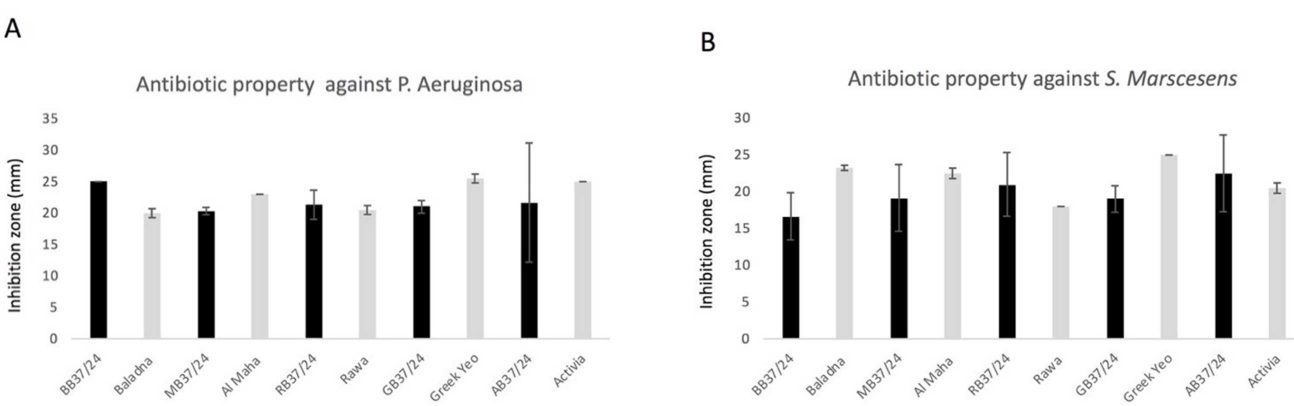

Figure 5. Antibiotic properties of commercial and lab synthetized yogurts

Table 3. T-test p-values for the yogurts' antibiotic property against *Pseudomonas aeruginosa*.

AP against Pseudomonas										
T-test p-values	BB37/24	MB37/24	RB37/24	GB37/24	AB37/24	Baladna	AlMaha	Rawa	GreekYeo	Ac-tivia
BB37/24	x	x	x	x	x	x	x	x	x	x
MB37/24	0.000	x	x	x	x	x	x	x	x	x
RB37/24	0.050	0.507	x	x	x	x	x	x	x	x
GB37/24	0.002	0.374	0.501	x	x	x	x	x	x	x
AB37/24	0.571	0.819	0.721	0.909	x	x	x	x	x	x
Baladna	0.001	0.789	0.537	0.591	0.879	x	x	x	x	x
Al Maha	0.000	0.008	0.676	0.075	0.862	0.038	x	x	x	x
Rawa	0.001	0.789	0.668	0.591	0.879	1.000	0.038	x	x	x
Greek Yeo	0.302	0.003	0.099	0.012	0.625	0.019	0.038	0.019	x	x
Activia	0.789	0.002	0.120	0.012	0.664	0.012	0.001	0.012	0.012	x

Before the intervention, the incubation of Baladna with *Pseudomonas aeruginosa* showed significantly smaller inhibition zones than using Al Maha, Yeo or Activia, and not significantly different than Rawa yogurt. However, after intervention using Baladna to make BB37/24, we observed significantly higher inhibition zones than Baladna itself, but also Al Maha and Rawa (Table 2, p=0.001; p=0.000; p=0.001). Moreover, the inhibition zone produced by BB37/24 was not significantly different than the Yeo yogurt or Activia. This suggests that the intervention has improved the antibiotic property of Baladna yogurt against *Pseudomonas aeruginosa*. Interestingly, we observed that the intervention using starters Al Maha and Yeo resulted in MB37/24 and GB37/24 with significantly smaller inhibition zones. The intervention was not effective in improving antibiotic property of yogurts Al Maha and Yeo. For *Serratia marscesens* (Figure 5, Table 4), the intervention resulted in yogurts inducing similar inhibition zones than their starters, except for GB37/24 where antibiotic property decreased significantly (p=0.02).

Susceptibility to Antibiotics

Before intervention, the Baladna yogurt showed significantly larger inhibition zones, suggesting higher susceptibility to streptomycin than Rawa, Yeo and Activia. Activia and Yeo had the least susceptibility to streptomycin (the most resistant) (Figure 6A, Table 5). The intervention significantly increased susceptibility to streptomycin of baladna (BB37/24) and Maha (MB37/24), but not the other yogurts. BB37/24 has a significantly higher susceptibility to streptomycin than all other yogurts.

Table 4: T-test p-values for the yogurts' antibiotic property against *Serratia marcesens*.

AP against Serratia										
T-test p-values	BB37/24	MB37/24	RB37/24	GB37/24	AB37/24	Baladna	Al Maha	Rawa	Greek Yeo	Activia
BB37/24	x	x	x	x	x	x	x	x	x	x
MB37/24	0.480	x	x	x	x	x	x	x	x	x
RB37/24	0.236	0.639	x	x	x	x	x	x	x	x
GB37/24	0.334	0.956	0.501	x	x	x	x	x	x	x
AB37/24	0.175	0.451	0.721	0.334	x	x	x	x	x	x
Baladna	0.071	0.314	0.537	0.052	0.860	x	x	x	x	x
Al Maha	0.095	0.399	0.676	0.087	1.000	0.312	x	x	x	x
Rawa	0.617	0.753	0.421	0.511	0.331	0.002	0.012	x	x	x
Greek Yeo	0.040	0.180	0.298	0.020	0.559	0.019	0.037	0.000	x	x
Activia	0.212	0.721	0.887	0.361	0.644	0.039	0.106	0.038	0.012	x

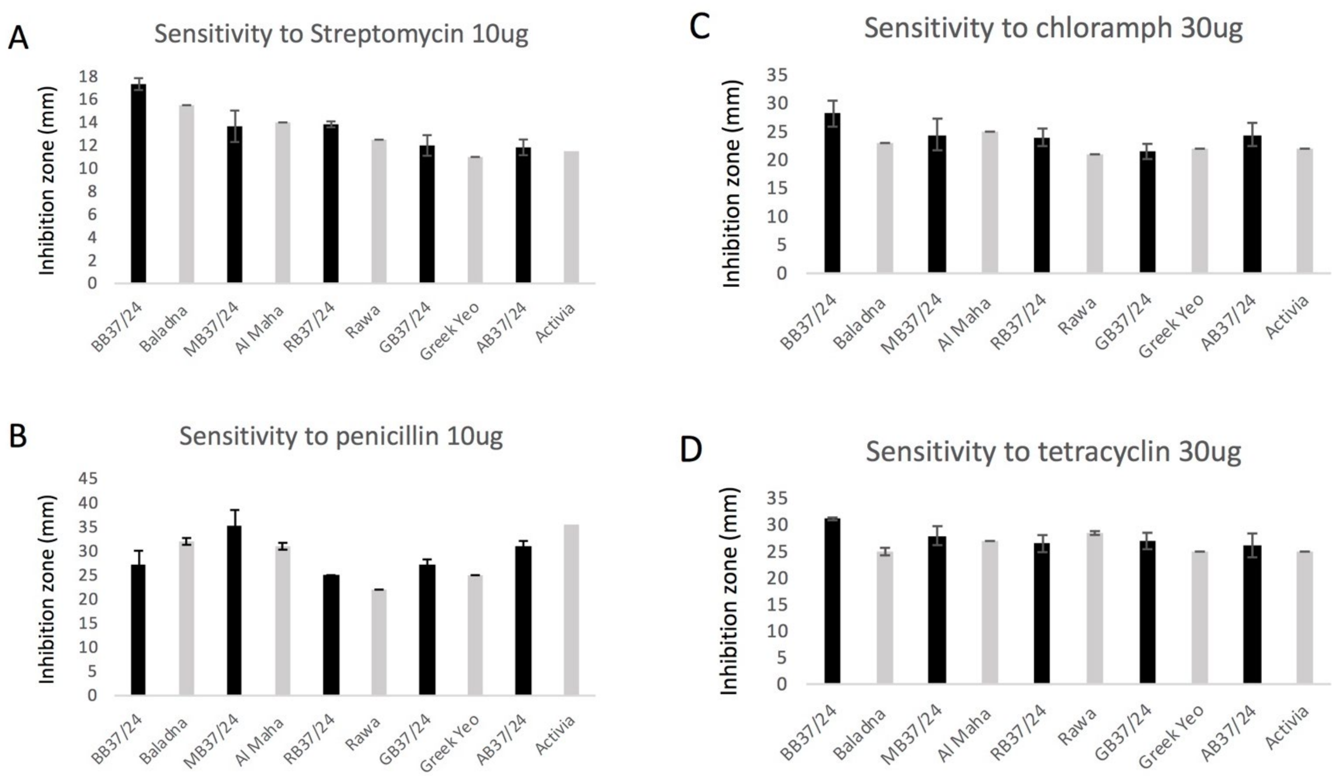

Figure 6. Susceptibility to antibiotics of commercial and lab synthetized yogurts.

Table 5: T-test p-values for the yogurts' antibiotic susceptibility to streptomycin.

T-test p-values	BB37/24	MB37/24	RB37/24	GB37/24	AB37/24	Baladna	Al Maha	Rawa	Greek Yeo	Activia
BB37/24	x	x	x	x	x	x	x	x	x	x
MB37/24	0.018	x	x	x	x	x	x	x	x	x
RB37/24	0.001	0.862	x	x	x	x	x	x	x	x
GB37/24	0.001	0.189	0.038	x	x	x	x	x	x	x
AB37/24	0.001	0.137	0.013	0.830	x	x	x	x	x	x
Baladna	0.021	0.264	0.016	0.024	0.011	x	x	x	x	x
Al Maha	0.016	0.537	0.219	0.058	0.030	0.312	x	x	x	x
Rawa	0.002	0.307	0.011	0.766	0.537	0.014	0.057	x	x	x
Greek Yeo	0.002	0.168	0.012	0.591	0.658	0.022	0.051	0.312	x	x
Activia	0.001	0.127	0.003	0.401	0.402	0.008	0.028	0.106	0.698	x

As for penicillin (Figure 6B, Table 6), Activia showed significantly larger inhibition zones than all other commercial yogurts, and Rawa was significantly the least susceptible.

Table 6: T-test p-values for the yogurts' antibiotic susceptibility to penicillin.

Penicillin										
T-test p-values	BB37/24	MB37/24	RB37/24	GB37/24	AB37/24	Baladna	Al Maha	Rawa	Greek Yeo	Activia
BB37/24	x	x	x	x	x	x	x	x	x	x
MB37/24	0.049	x	x	x	x	x	x	x	x	x
RB37/24	0.256	0.009	x	x	x	x	x	x	x	x
GB37/24	1.000	0.025	0.132	x	x	x	x	x	x	x
AB37/24	0.109	0.150	0.778	0.661	x	x	x	x	x	x
Baladna	0.169	0.169	0.495	0.483	0.724	x	x	x	x	x
Al Maha	0.264	0.197	0.131	1.000	0.495	0.293	x	x	x	x
Rawa	0.101	0.018	0.272	0.409	0.001	0.003	0.003	x	x	x
Greek Yeo	0.307	0.032	0.272	0.046	0.004	0.010	0.014	0.038	x	x
Activia	0.040	0.978	0.000	0.002	0.014	0.022	0.014	0.000	0.003	x

The intervention did not significantly change susceptibility of the yogurts to penicillin, except for Activia which became lower. For chloramphenicol (Figure 6C, Table 7), most yogurts had similar inhibition zones. The intervention did not significantly change susceptibility of the yogurts to chloramphenicol.

Table 7: T-test p-values for the yogurts' antibiotic susceptibility to chloramphenicol.

Chloramphenicol										
T-test p-values	BB37/24	MB37/24	RB37/24	GB37/24	AB37/24	Baladna	Al Maha	Rawa	Greek Yeo	Activia
BB37/24	x	x	x	x	x	x	x	x	x	x
MB37/24	0.191	x	x	x	x	x	x	x	x	x
RB37/24	0.031	0.389	x	x	x	x	x	x	x	x
GB37/24	0.109	0.109	0.132	x	x	x	x	x	x	x
AB37/24	0.139	1.000	0.778	0.131	x	x	x	x	x	x
Baladna	0.074	0.565	0.495	0.272	0.444	x	x	x	x	x
Al Maha	0.202	0.828	0.476	0.050	0.769	0.001	x	x	x	x
Rawa	0.034	0.235	0.235	0.714	0.137	0.001	0.000	x	x	x
Greek Yeo	0.042	0.293	0.293	1.000	0.185	0.095	0.019	0.465	x	x
Activia	0.049	0.370	0.370	0.657	0.247	0.003	0.001	0.005	0.388	x

The yogurts with significantly largest inhibition zones were BB37/24 and AB37/24. Finally, for tetracyclin (Figure 6D, Table 8), before intervention, the commercial yogurts Al Maha and Rawa showed significantly larger inhibition zone than the probiotic yogurts Yeo and Activia.

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Table 8: T-test p-values for the yogurts' antibiotic susceptibility to tetracyclin.

Tetracyclin										
T-test p-values	BB37/24	MB37/24	RB37/24	GB37/24	AB37/24	Baladna	Al Maha	Rawa	Greek Yeo	Activia
BB37/24	x	x	x	x	x	x	x	x	x	x
MB37/24	0.053	x	x	x	x	x	x	x	x	x
RB37/24	0.011	0.389	x	x	x	x	x	x	x	x
GB37/24	0.015	0.548	0.746	x	x	x	x	x	x	x
AB37/24	0.027	0.379	0.861	0.661	x	x	x	x	x	x
Baladna	0.001	0.107	0.247	0.160	0.448	x	x	x	x	x
Al Maha	0.000	0.550	0.735	1.000	0.687	0.038	x	x	x	x
Rawa	0.002	0.878	0.287	0.409	0.350	0.312	0.038	x	x	x
Greek Yeo	0.000	0.142	0.360	0.228	0.594	0.038	0.001	0.006	x	x
Activia	0.000	0.138	0.346	0.219	0.578	0.539	0.001	0.006	0.423	x

The Baladna yogurt showed significantly smaller inhibition zones than all other yogurts. After intervention using the Baladna yogurt, BB37/24 showed significantly larger inhibition zones than all other yogurts, suggesting that the protocol was successful in improving resistance to tetracyclin making the yogurt less resistant. This was only observed for Baladna and no significant change was observed when using the other starters.

Discussion

Probiotic content of yogurts depends on several variables, including temperature of incubation, duration of the incubation, amount of oxygen present during incubation, the type of starter and milk used, and additives added if any. Therefore, every yogurt will be almost unique. In this study, we decided to focus on the effect of the starter, while keeping the other parameters the same. This study compares various characteristics of commercial yogurts before and after intervention, which consisted in a 24 hours-based fermentation at 37 C.

Enhancement of the Resistance to Acidity

In order for probiotic bacteria to inhibit pathogens in the gut, they need to resist to the acidity produced by the gastrointestinal tract. A test was performed to assess survival of the bacteria after exposure to three hours of acidity. The intervention resulted in a ten-fold increase in CFU/ml count for Baladna yogurt (BB37 vs Baladna, $p<0.0002$). Significant increase was also observed for the Rawa yogurt (RB37 vs Rawa, $p= 0.007$), and Activia (AB37 vs Activia, $p=0.05$). The difference was not significant when starting with Al Maha or Yeo yogurts. Interestingly, these two yogurts, when used as starters, produced yogurts with similar probiotic profiles (S1). The taxonomic data showed that these yogurts are more abudnant in *Bifidobacterium animalis* than the other yogurts. Furthermore, it was shown previously that *Bifidobacterium animalis* grows better at 42 °C [33]. While the BSM agar did not allow growth in all yogurts, th M17 has was chosen over the nutrient agar and the BSM agar as it was the only one that allowed growth.

However, because Al Maha contains more of *Lactococcus lactis* and *Bifidobacterium animalis,* the agar and broth used in our colony counting might not have been optimal to allow growth of these bacteria.

The two yogurts with highest resistance to the acidity test were the ones produced using Baladna or Rawaa as starters. The CFU/ml counts in these two yogurts were significantly higher than probiotic yogurts of the market such as Activia or Yeo. While resistance to acids was demonstrated, future studies should include a tolerance test to bile acids as well.

Diversity

All yogurts produced had *Streptococcus Thermophilus* as predominant bacteria. In fact, when using Activia, which did not initially have *Streptococcus Thermophilus* as the most abundant bacteria, the protocol used also produced a yogurt with a higher abundance of *Streptococcus Thermophilus*, which suggests that the specific parameters used promote the growth of this species.

However, each of the yogurts made was also characterized by the particular abundance of one or two other species. RB37/24 is the only yogurt with significantly higher abundance of *Lactococcus Lactis* than all other yogurts made or even the commercial yogurts tested. AB37/24 made using Activia as a starter showed significantly higher abundance of *Lactobacillus Bulgaricus* than all other yogurts, which is not surprising as the commercial Activia has also shown a significantly higher abundance of this bacterium than all other commercial yogurts tested. Yogurts produced using Al Maha or Yeo yogurts as starters seem to have similar profiles in term of abundance of each of these four bacterial types. Nevertheless, latest human microbiome studies are shifting toward the importance of diversity rather than abundance of specific microbes [34]–[36]. Studies have started to focus more on metabolomics than phylogenetic analyses, arguing that metabolites produced by microbes are the real determinants in the gut microbiome to health relationship [37].

Antibiotic Properties

Serratia Marscesens and *Pseudomonas aeruginosas* are responsible for nosocomial infections. This study shows that overall, the newly developed yogurts have similar action against these pathogens as commercial yogurts, including the probiotic yogurts Activia and Yeo. We note that while the commercial Baladna yogurt has lower antibiotic properties as compared to Activia, the newly developed yogurt using Baladna as a starter shows actually the same antibiotic property as Activia against *Pseudomonas aeruginosa*. The inhibition zone of 25 mm corresponds to the inhibition done by 30 µg of Aztreonam [38]. The smallest inhibition zone produced by all yogurts was 20 mm for *Pseudomonas*, which corresponds to the inhibition done by 10µg of Imipenem, and better than inhibition done by 30 µg of Cefotaxime or 30 µg Ceftriaxone [38]. While some have suggested a role for Yakult as an efficient intervention to treat multidrug resistant *Pseudomonas* infections [39], this study emphasizes the potential of probiotic yogurts in fighting this pathogen and provides new options.

Because the local commercial yogurts were never characterized before, it is important to note here that the local yogurts Baladna and Al Maha are showing similar antibiotic properties against *Serratia Marscesens*, if not better, than the probiotic yogurts Activia and Yeo. As for the activity against *Pseudomonas*, the antibacterial activity of local yogurts is slightly less than Yeo and Activia but remains better than some antibiotics [38]. Indeed, the smallest inhibition found was 17mm for Serratia Marcescens, and this equivalent to 2 to 5 µg of loading a disc with ceftazidime [40], the last trend in treating *Serratia Marscesens* infections [41].

Susceptibility to Antibiotics

With the rising incidence of antimicrobial resistance and limited number of discovered antibiotic compounds, the increase of multidrug resistance is an imminent threat. Considering that probiotic microbes are beneficial for health, their resistance to antibiotics could be seen as an advantage. However, resistance genes can be passed on by conjugation to other microbes and therefore, if these products were to be consumed daily, that would mean ingesting resistant bacteria daily and increasing the risk of passing on resistant genes to more pathogenic microbes within the gut microbiota. For this reason, it is essential to investigate on the antibiotic resistance of these yogurts in comparison to available yogurts in the market.

One of the first observations is that the protocol presented, which uses a 24 fermentation at 37°C results in yogurts (with the exception of MB37/24), newly developed yogurts are significantly less resistant to streptomycin than their respective starters in the market. The BB37/24 is significantly less resistant to tetracyclin than its starter. Interestingly, the yogurt that showed the least resistance to penicillin is MB37/24. Overall, Qatar's newly commercialized yogurts are either equivalent or less resistant to antibiotics than Activia and Yeo.

While this article advocates for the improved qualities of the 24h fermented version of yogurts, these data are not sufficient to infer long-term effects of daily consumption of probiotic yogurts. In fact, we are hoping with this article to emphasis at how versatile outcomes can result from simply changing the starter and duration of incubation. Further studies should focus on increasing sample sizes and comparing antibiotic resistance of commercial and newly developed products against more antibiotics.

Conclusion

This study shows that two of Qatar's newly commercialized plain yogurts, Baladna and Rawa, have significantly better resistance to acidity than Activia and/or Yeo and contain enough probiotic bacteria to be labelled as probiotics. However, like most of commercial yogurts, they may contain additives such as skimmed milk powder, whey powder, and larger amount of lactose because of incomplete fermentation, which might not be suitable to all (Kalit et al, 2008). In this study, we have used a protocol to attempt to enhance some of the characteristics of these yogurts using a 24h fermentation and without any additives and no sugar, a solution for people with intolerances. Results of the intervention comparing each produced yogurt to its starter for the characteristics explored are summarized in Table 9.

The BB37/24, under the stated growth conditions, showed overall best results with highest resistance to acidity then all yogurts including Activia, the highest abundance of *Streptococcus thermophilus* of all yogurts, the highest antibiotic property against *Pseudomonas aeruginosa* among the newly developed yogurts, the least resistance to streptomycin, to chloramphenicol and tetracyclins out of all yogurts. However, these need to be reproduced using different agar media and concentrations to further confirm these statements.

The method is easily reproducible as a homemade yogurt, using commercially available local baladna as a starter, which is much cheaper than imported yogurts. We expect that adding prebiotic fibers would increase the CFU/ml counts, and mixing starters could eventually lead to more diverse probiotic content. Further explorations should aim at exploring lactose content of the 24 hours fermented yogurts, properties against other pathogens, resistance to other antibiotics and assessing benefits and risks of daily ingestion of probiotics.

Table 9. Summary of effects of the intervention. T-test p-values comparing each yogurt to its starter. Arrows up show a significant increase after intervention, arrows down show a significant decrease after intervention.

	Baladna vs BB37/24	**Maha vs MB37/24**	**Rawa vs RB37/24**	**Yeo vs GB37/24**	**Activia vs AB37/24**
CFU count/ml in the yogurt	0.127	0.336	0.006 ↑	0.003 ↓	0.332
Resistance to acidity (CFU/ml)	0.000 ↑	0.785	0.070	0.117	0.177
Antibiotic property against Pseudomonas	0.001 ↑	0.008 ↓	0.668	0.012 ↓	0.664
Antibiotic property against Serratia	0.071	0.399	0.421	0.020 ↓	0.644
Antibiotic resistance (less resistance, more sensitive) against streptomycin	0.021↓	0.537	0.011↓	0.591	0.402
Antibiotic resistance (less resistance, more sensitive) against penicillin	0.169	0.197	0.272	0.046↓	0.014↑
Antibiotic resistance (less resistance, more sensitive) against chloramphenicol	0.074	0.235	0.235	1.000	0.247
Antibiotic resistance (less resistance, more sensitive) against tetracyclin	0.001↓	0.550	0.287	0.228	0.578

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