



Antagonistic effect of developed probiotic yoghurt against some selected food-borne pathogens during cold storage

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ABSTRACT

This study was carried out to investigate the antagonistic effect of probiotic yoghurt against some selected food-borne pathogens during cold storage. Probiotic yoghurt was produced from processed cow milk using controlled fermentation, and later inoculated with selected food-borne pathogens at inoculum level of 10^5 CFU/mL. The antagonistic effect of the probiotic yoghurt against the food-borne pathogens during cold storage (4°C) for 2 days was studied using standard methods. The results obtained demonstrated that the probiotic yoghurt inhibited growth of food-borne pathogens including *Salmonella typhimurium* ATCC13311, *Proteus mirabilis* ATCC25933, and *Escherichia coli* ATCC2592 in the yoghurt within 24 hr. Their counts decreased from 10^5 - 10^2 CFU/mL, with pH and probiotic counts ranging between 4.48-4.35, and 10^6 - 10^8 CFU/mL, respectively. The probiotic bacteria have the ability to suppress the growth of pathogen like *E. coli* in yoghurt. This inhibitory effect may be due to low pH of yoghurt. The probiotic cultures in the yoghurt can be used as biopreservatives in food and pharmaceutical industries.

INTRODUCTION

Lactic acid bacteria are Gram positive rods or cocci, which have the ability to produce lactic acid. Lactic acid bacteria (LAB) could be used successfully with limited or no negative effects, to control challenging problems associated with some enterics. The substances produced by the LAB, are kept in the foods which could help to inhibit pathogens (Brant and Todd, 2014; Mohammed et al., 2016). The antimicrobial potential of LAB can be due to ability in producing substances like lactic acid which has the tendency to suppress pathogenic microbes (Brant and Todd, 2014; Tribe et al., 2014; Nikolic et al., 2008). In addition to production of organic acids, the pH reduction of the products or medium can have antagonistic effect on pathogens.

Some studies have reported that strains of *Salmonella typhimurium*, and other pathogens were suppressed by substances produced by LAB ((Brant and Todd, 2014; Tribe et al., 2014; Evariste et al., 2017; Gopalakrishnan, 2018). Therefore, the probiotic LAB make the environment unfavourable for pathogens to thrive during the manufacture of probiotic yoghurt, hence limiting the viability of the pathogens (Mohammed et al., 2016; Ting and Xialian, 2019). Moreover, milk and milk products are usually affected by pathogen like *E. coli* and *Salmonella typhimurium*, since some strains can survive acidic conditions (Bibbal et al., 2014; Rebello et al., 2014; CDC, 2016; Evariste et al., 2017). However, there is little attention on the survival of these pathogens in yoghurt. Therefore, there is a need to assess the antagonistic effect of probiotic yoghurt against food-borne pathogens during cold storage.

MATERIALS & METHODS

Collection of samples

Raw milk from white Fulani cow was purchased from Dairy and Research Farm, University of Ibadan, Ibadan, Nigeria. It was brought into Physiology Laboratory, at the Department of Microbiology in sterile bottles for production of yoghurt.

Collection of Indicator organisms

Indicator organisms such as *Salmonella typhimurium* ATCC13311, *Proteus mirabilis* ATCC25933, and *Escherichia coli* ATCC25922 were obtained from the culture collection unit of Federal Institute of Industrial Research, Oshodi (FIIRO).

Preparation of inoculum size of pathogens

Each strain of *Salmonella typhimurium* ATCC13311, *Proteus mirabilis* ATCC25933 and *Escherichia coli* ATCC25922 were inoculated in 10 mL tryptic soya broth containing 0.6 % yeast extract, and

incubated at 37°C for 24 hr. The serial dilutions was made, and the inoculation level was determined by direct plating on specific media of the pathogens from serial dilution of broth. Inoculum size of 10⁵ CFU/mL was used (ISO, 2003).

Probiotic cultures

Potential probiotic starters such as *Lactobacillus plantarum*N24, *Lactobacillus plantarum*N17, *Lactobacillus brevis* N10, and *Lactobacillus casei*N1 isolated from *nono* samples were used to produce yoghurt.

Antagonistic effect of probiotic yoghurt against food-borne pathogens

Yoghurt was prepared in the laboratory using method described by Rahmann et al. (1999). For each yoghurt samples, 100 mL of the cow milk was heated to 85°C for 30 minutes, and then cooled immediately in an ice bath to temperature of 37°C. This was then, inoculated with 10⁶ CFU/mL probiotic starters, and incubated at 42°C for 4 hr. After yoghurt formation, yoghurt was inoculated with the pathogens at inoculation size of 10⁵ CFU/mL. The yoghurt without inoculation of pathogens (control), and the inoculated yoghurt were stored in the refrigerator. The yoghurt samples were examined microbiologically for pathogens count during 2 days of storage, using pour plate technique in Petri dishes. The viability of probiotic cultures and pathogenic organisms was done every 24 hr during 2 days of storage according to methods of ISO (2003) and (ISO, 2004), respectively. One mL of appropriate dilutions of yoghurt samples were plated on Mac Conkey agar (for *Proteus*), Eosin methylene blue agar (for *E. coli*), Salmonella Shigella agar for *Salmonella*, and MRS agar for lactic acid bacteria, and incubated at 37°C for 48 hrs. Colony forming unit were then estimated. The pH was determined using a pH meter, following the manufacturer's instructions (APHA, 2004). The experiments were done in duplicates.

Statistical analysis

The values for each parameters were calculated and presented as means of duplicates. Data was analysed using Analysis of Variance (ANOVA) with Duncan Multiple Range Test for significance at P≤0.05. Standard deviation was not shown. Data were also presented in tables.

RESULTS AND DISCUSSION

The codes of prepared probiotic yoghurt with pathogens and without pathogens are shown in Table 1. Table 2 showed the effect of probiotics against *Proteus mirabilis* ATCC 25933, during storage period at 4°C for 2 days, when the initial inoculum size of the pathogen was

10^5 CFU/mL. At first day of storage, the count of *Proteus mirabilis* reduced from 10^5 to 10^3 and 10^2 CFU/mL, and their counts were significantly different ($P \leq 0.05$) in probiotic yoghurt samples, when pH ranged from 4.40-4.45. By the second day, the inoculated *Proteus mirabilis* was not found in the probiotic yoghurt, when probiotic cultures count was between 10^6 to 10^8 CFU/mL, and pH ranged between 4.34- 4.42.

However, similar studies also demonstrated that the pathogen was not detected after 48 hr of storage period as reported by Bachrouri et al. (2006). This could be as a result of increased probiotic cultures count, pH and probiotic strain used to produce yoghurt. Our findings are in accordance with the work of Ting and Xialian. (2019) in terms of inhibition at 24 hr of cold storage. They studied antagonistic effect on *Proteus*

mirabilis, and some spoilage organisms in yoghurt fermented with probiotic starters, these pathogens were inhibited completely within 24-48 hr. The inhibition of pathogens could be as a result of probiotic starters strain used to produce yoghurt (Wang et al., 2004).

Table 3 showed the antagonistic effect of probiotic cultures against *Salmonella typhimurium* ATCC13311 during the cold storage (4°C) for 2 days with the initial inoculum size of *Salmonella typhimurium* ATCC13311 at 10^5 CFU/mL. At first day of storage, the initial count of the pathogen decreased from 10^5 - 10^2 CFU/mL, but not found in sample YN24-N17, when the pH was 4.38. At second day, *Salmonella typhimurium* disappeared in sample YN17, with pH 4.37, and probiotic count (2.0×10^7 CFU/mL), which increased to 10^8 CFU/mL.

Table 1: Codes of prepared probiotic yoghurt

Samples	Prepared probiotic yoghurt (inoculated with pathogens)	Prepared probiotic yoghurt (without pathogens)
1	YN24	yn24
2	YN17	yn17
3	YN10	yn10
4	YN1	yn1
5	YN24-N17	yn24-n17
6	YN24-N10	yn24-n10
7	YN24-N1	yn24-n1
8	YN17-N10	yn17-n10
9	YN17-N1	yn17-n1
10	YN10-N1	yn10-n1

*Samples with Capital letters codes (prepared probiotic yoghurt inoculated with pathogens)

*Samples with small letters codes (prepared probiotic yoghurt without pathogens)

The experiment was done in duplicates

Keys:

1-Yoghurt made from cow milk and *Lactobacillus plantarum*N24

2-Yoghurt made from cow milk and *Lactobacillus plantarum*N17

3 -Yoghurt made from milk and *Lactobacillus brevis*N10

4 -Yoghurt made from cow milk and *Lactobacillus casei*N1

5-Yoghurt made from cow milk and *Lactobacillus plantarum*N24 & *Lactobacillus plantarum*N17

6-Yoghurt made from cow milk and *Lactobacillus plantarum*N24 & *Lactobacillus brevis*N10

7-Yoghurt made from cow milk and *Lactobacillus plantarum*N24 & *Lactobacillus casei*N1

8-Yoghurt made from cow milk and *Lactobacillus plantarum*N24 & *Lactobacillus brevis*N10

9-Yoghurt made from cow milk and *Lactobacillus plantarum*N17 & *Lactobacillus casei*N1

10-Yoghurt made from cow milk and *Lactobacillus brevis*N10 & *Lactobacillus casei*N1,

Table 2: Antagonistic effect of developed probiotic yoghurt against of *Proteus mirabilis* ATCC25933 stored under cold storage (4°C)

Samples Codes	Storage time(days)		Bacterial Count(CFU/mL)		
	1	2	1	2	3
	pH	PMC	pH	PMC	PCC
YN24	4.42 ^b	2.1x10 ^{3a}	4.40 ^a	-	5.0 x10 ^{6c}
yn24*	4.40 ^c	-	4.39 ^{ab}	-	1.2x10 ^{7b}
YN17	4.43 ^{ab}	2.3x10 ^{3a}	4.42 ^a	-	6.0 x10 ^{6b}
yn17	4.40 ^c	-	4.40 ^a	-	6.9x10 ^{6b}
YN10	4.45 ^a	3.5x10 ^{3a}	4.39 ^{ab}	-	1.2x10 ^{7b}
yn10	4.43 ^{ab}	-	4.39 ^{ab}	-	6.4x10 ^{6b}
YN1	4.44 ^a	1.7x10 ^{3a}	4.40 ^a	-	5.8x10 ^{6b}
yn1	4.41 ^c	-	4.38 ^{ab}	-	6.7x10 ^{6b}
YN24-N17	4.41 ^c	1.8x10 ^{2b}	4.38 ^{ab}	-	2.0x10 ^{7b}
yn24-n17	4.40 ^c	-	4.38 ^{ab}	-	3.1x10 ^{7b}
YN24-N10	4.40 ^c	1.2x10 ^{2b}	4.36 ^b	-	1.8x10 ^{7b}
yn24-n10	4.39 ^c	-	4.36 ^b	-	3.7x10 ^{7b}
YN24-N1	4.42 ^b	3.0x10 ^{2ab}	4.38 ^{ab}	-	1.5x10 ^{7b}
yn24-n1	4.41 ^c	-	4.36 ^b	-	2.7x10 ^{7b}
YN17-N10	4.40 ^c	1.2x10 ^{2b}	4.35 ^b	-	1.3x10 ^{7b}
yn17-n10	4.40 ^c	-	4.37 ^{ab}	-	1.4x10 ^{8a}
YN17-N1	4.42 ^b	1.2x10 ^{2b}	4.39 ^{ab}	-	2.0x10 ^{7b}
yn17-n1	4.43 ^{ab}	-	4.37 ^{ab}	-	3.5x10 ^{7b}
YN10-N1	4.40 ^c	1.3x10 ^{2b}	4.34 ^b	-	1.8x10 ^{7b}
yn10-n1	4.42 ^b	-	4.35 ^b	-	2.8x10 ^{7b}

Means with the same alphabets within a column are not significantly different at $P \leq 0.05$ using Duncan Multiple Range Test (DMRT). Data collected were represented as "Means of duplicates. Standard Deviation (SD)" not shown., - = Not viable, initial and inoculum size of *Proteus mirabilis* = 10^5 CFU/mL, PMC = *Proteus mirabilis* count, PCC = Probiotic cultures count, inoculum size of starters = 10^6 CFU/mL, PCC for the first day = all were 10^6 CFU/mL.

*All small letters (yoghurt without pathogen) Samples with capital letters (yoghurt inoculated with *Proteus mirabilis* ATCC25933)

This is in accordance with the work of Al-Delanmy and Hamamdeh (2013) that reported the inhibition of *Salmonella typhimurium* during 48 hr of cold storage. The reasons for suppression of pathogen could be low pH, and inability to compete with the probiotic cultures for nutrients (Wang et al., 2004; Tsegaye and Ashenafi, 2005; Donkor et al., 2006; Gopalakrishnan, 2018, Nassib et al., 2006, Ting and Xialian, 2019). Probiotics LAB could prevent growth of pathogens due to low pH. The variation observed by various scientists might be due to difference in survival of strain to lowered pH and

temperature, type and strain of starter cultures being used, inoculum size of starters, and the pathogens (Tsegaye and Ashenafi, 2005). The probiotic bacteria have the ability to prevent the growth of pathogens due to low pH initiated by LAB. The fermentation time and temperature, type of probiotic organisms, increased probiotic cultures count, acid tolerance, and the strain of the pathogenic organisms could play important role on the survival of food pathogens in yoghurt. Donkor *et al.* (2006) concluded that the ability of probiotic to survive in yoghurt was strain dependent.

Table 3: Antagonistic effect of developed probiotic yoghurt against *Salmonella typhimurium* ATCC13311 during cold storage (4°C).

Samples Codes	Storage time (days)		Bacterial count (CFU/mL)		
	1	2	1	2	2
	pH	STC	pH	STC	PCC
YN24	4.40 ^{ab}	3.3x10 ^{3a}	4.39 ^{ab}	-	1.0 x10 ^{7b}
n24 *	4.40 ^a	-	4.38 ^{ab}	-	1.9x10 ^{7b}
YN17	4.42 ^a	2.3x10 ^{3a}	4.37 ^b	-	2.0 x10 ^{7b}
yn17	4.40 ^a	-	4.37 ^b	-	2.5x10 ^{7b}
YN10	4.41 ^a	1.9x10 ^{3a}	4.38 ^{ab}	-	3.0x10 ^{7b}
yn10	4.41 ^a	-	4.37 ^{ab}	-	3.6x10 ^{7ab}
YN1	4.41 ^a	1.4x10 ^{3a}	4.40 ^a	-	3.3x10 ^{7b}
yn1	4.40 ^a	-	4.38 ^{ab}	-	3.8x10 ^{7ab}
YN24-N17	4.38 ^{ab}	-	4.36 ^b	-	1.1x10 ^{8a}
Yn24-n17	4.36 ^b	-	4.36 ^b	-	1.9x10 ^{8a}
Y24-N10	4.36 ^b	-	4.35 ^b	-	1.3x10 ^{8a}
yn24-n10	4.35 ^b	-	4.35 ^b	-	1.6x10 ^{8a}
YN24-N1	4.40 ^a	1.2x10 ^{2b}	4.39 ^{ab}	-	2.5x10 ^{7b}
yn24-n1	4.39 ^{ab}	-	4.37 ^b	-	2.7x10 ^{7b}
YN17-N10	4.37 ^{ab}	-	4.36 ^b	-	1.1x10 ^{8a}
yn17-n10	4.36 ^b	-	4.36 ^b	-	2.2x10 ^{8a}
YN17-N1	4.41 ^a	1.5x10 ^{2b}	4.39 ^{ab}	-	3.2x10 ^{7b}
yn17-n1	4.40 ^a	-	4.38 ^{ab}	-	3.7x10 ^{7ab}
YN10-N1	4.37 ^{ab}	-	4.35 ^b	-	1.4x10 ^{8a}
yn10-n1	4.36 ^b	-	4.35 ^b	-	1.9x10 ^{8a}

Means with the same alphabets within a column are not significantly different at $P \leq 0.05$ using Duncan Multiple Range Test (DMRT). Data collected were represented as "Means of duplicates. Standard Deviation (SD)" not shown.

- = Not viable, initial and inoculum size of *Salmonella typhimurium* = 10^5 CFU/mL, STC = *Salmonella typhimurium* count, PCC = Probiotic cultures count, inoculum size of Probiotic starters and PCC at the first day = 10^6 CFU/mL,

*All small letters (yoghurt without pathogens),

*Samples with capital letters (yoghurt inoculated with *Salmonella typhimurium* ATCC13311)

Table 4 showed that *E. coli* ATCC29522 was suppressed completely at the first day of storage in sample like YN24-N17, with pH of 4.36, and inhibited from 10^5 to 10^2 CFU/mL in sample YN24. The decrease of the initial count of *E. coli* ATCC29522 from 10^5 - 10^2 CFU/mL illustrates the antagonistic effect of the probiotic cultures on the pathogenic organisms which was due to pH and higher viable count of probiotics.

However, *E. coli* ATCC29522 completely disappeared at the second day of storage with increased probiotic counts of 10^8 CFU/mL, when pH of

probiotic yoghurt samples were not significantly different from each other at $P \leq 0.05$. A similar study was reported by Kasimoglu and Akgun (2004), indicating that there was total suppression of *E. coli* within 48 hr after storage of the milk inoculated with 10^6 CFU/mL of probiotics. This could be due to the low pH below 4.39. The variable results of most authors could be strain dependent. Moreover, acid survival of food pathogens and their acid adaptation can enhance the survival of these organisms in acidic foods like yoghurt during fermentation.

Table 4: Antagonistic effect of probiotic yoghurt against *Escherichia coli* ATCC25922 (10⁵ CFU/mL) under cold storage (4°C)

Samples	Storage time(days)		Bacterial count(CFU/mL)		
	1	2			
Codes	pH	ECC	pH	ECC	PCC
YN24	4.40 ^a	1.4x10 ^{3a}	4.37 ^a	-	2.4x10 ^{7b}
yn24 *	4.40 ^a	-	4.36 ^a	-	2.9x10 ^{7b}
YN17	4.41 ^a	2.4x10 ^{3a}	4.37 ^a	-	1.4 x10 ^{7b}
yn17	4.40 ^a	-	4.37 ^a	-	2.9x10 ^{7b}
YN10	4.39 ^{ab}	1.8x10 ^{2b}	4.37 ^a	-	3.2x10 ^{7b}
yn10	4.39 ^{ab}	-	4.36 ^a	-	3.8x10 ^{7b}
YN1	4.40 ^{ab}	1.7x10 ^{3a}	4.39 ^a	-	3.6x10 ^{7b}
yn1	4.38 ^{ab}	-	4.38 ^a	-	4.4x10 ^{7b}
YN24-N17	4.37 ^b	-	4.36 ^a	-	2.1x10 ^{8a}
yn24-n17	4.36 ^b	-	4.36 ^a	-	2.9x10 ^{8a}
YN24-N10	4.36 ^b	-	4.35 ^a	-	1.8x10 ^{8a}
yn24-n10	4.36 ^b	-	4.36 ^a	-	2.6x10 ^{8a}
YN24-N1	4.37 ^b	-	4.36 ^a	-	1.5x10 ^{8a}
yn24-n1	4.36 ^b	-	4.35 ^a	-	2.7x10 ^{8a}
YN17-N10	4.37 ^b	-	4.35 ^a	-	2.4x10 ^{8a}
yn17-n10	4.35 ^b	-	4.35 ^a	-	2.9x10 ^{8a}
YN17-N1	4.36 ^b	-	4.35 ^a	-	1.6x10 ^{8a}
yn17-n1	4.35 ^b	-	4.34 ^a	-	1.7x10 ^{8a}
YN10-N1	4.36 ^b	-	4.35 ^a	-	3.2x10 ^{7a}
yn10-n1	4.35 ^b	-	4.35 ^a	-	1.5x10 ^{8a}

Means with the same alphabets within a column are not significantly different at P≤0.05 using Duncan Multiple Range Test (DMRT). Data collected were represented as "Means of duplicates. Standard Deviation (SD)" not shown.

- = Not viable, initial and inoculum size of *E. coli* ATCC25922= 10⁵ CFU/mL,

ECC = *E. coli* count, PCC=Probiotic cultures count, inoculum size of starters=10⁶ CFU/mL,

*All small letters samples (yoghurt without pathogen)

*Samples with capital letters (yoghurt inoculated with *E. coli* ATCC25922).

CONCLUSIONS

The probiotic starters in the yoghurt were able to inhibit the growth, and suppressed selected food borne pathogens within a short period of 24 hr, suggesting that better ones could be useful to prevent or treat illness caused by pathogenic organisms, and also as preservatives in food and pharmaceutical industries.

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