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4 **BIOCHEMICAL CHARACTERIZATION OF MICROBIAL ISOLATES AND EFFECT**
5 **OF CONCENTRATION AND INCUBATION TIME OF PLANT AND COW MILK**
6 **YOGHURT USING RESPONSE SURFACE METHODOLOGY.**
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10 **ABSTRACT:**

11 The objective of the research was to evaluate the biochemical characteristics of the various microbial
12 isolates used in the production of plant based yoghurt, the optimal growth temperatures and the effect of
13 incubation time and inoculum concentration on the taste and desirability of the yoghurt. Pure cultures of
14 five (5) microbial strains (*Lactobacillus acidophilus*; *Lactobacillus bulgaricus*; *Lactobacillus casei*;
15 *Lactobacillus lactis*; *Streptococcus thermophilus*) were obtained from commercial yoghurt and evaluated
16 for their biochemical characteristics, bacterial growth temperatures and syneresis. The isolated organisms
17 were used to ferment plant-based milk products produced from blends of tigernut, dates and coconut.
18 Response Surface Methodology (Face centered composite design) was used to obtain an optimized ratio
19 formulation of 0.167 for coconut, 0.667 for tigernut and 0.167 for dates. Bacterial growth temperatures
20 were evaluated at 4°C, 27°C, 37°C, 40°C, 45°C and 60°C, respectively. Results showed that full bacterial
21 growth were noticed at temperatures of 40-45°C. The taste of the yoghurt improved from 6.58
22 to 6.71 when the incubation temperature changed from about 45°C to 44°C, and the duration of incubation
23 changed from 15.4hrs to 14.7hrs.

24 **KEYWORDS:** Yoghurt, Inoculum Concentration, Tigernut, Dates, Coconut.

25
26 **1. INTRODUCTION:**

27 Production of yoghurt have been carried out over the years using bacterial cultures such as
28 *Lactobacillus bulgaricus* and *Streptococcus thermophilus*. The fermentation process involved in
29 yoghurt production leads to a decrease in pH and the development of enhanced organoleptic
30 properties [1]. Dairy milk has been the primary source of yoghurt product, however there is an
31 increasing need of plant-based yoghurt products due to the health benefits associated with them.
32 Makinen [2] reported that plant-based yoghurts are generally made from fermenting aqueous
33 extracts obtained from different plant raw materials such as legumes, Cereals and Oil seeds. The
34 use of Plant based alternatives can be added to the high cost of dairy based products and the
35 presence of α -lactoglobulin and β -lactoglobulin, which have been shown to cause food allergy in
36 some individuals [3]. The problem of lactose intolerance, a condition caused by the inability of
37 the individual to digest lactose, has become a factor limiting the use of dairy milk and its product
38 [4]. The consumption of probiotic yoghurts have been shown to have functional benefits for
39 the consumers. Probiotics have been reported to play a role in reducing the occurrence, duration
40 and severity of flu [5]. Various investigations have been carried out on the possibility of using
41 plant based milk sources such as coconut, soybean and tigernut as an alternative to dairy milk
42 [6,7,8]. Milk extracted from tiger nut, apart from being nutritious, has been recommended for
43 persons that do not tolerate gluten or are allergic to cow milk and its derivatives [9].

44 Tigernut milk is very popular in Nigeria. The milk is a viscous cream coloured liquid with a very
45 short shelf-life (48 h) when produced traditionally [10]. It has been reported to be of great health
46 benefits. It is rich in mineral content and aids bones, tissue repair, muscles, the bloodstream and
47 body development [11]. Other health benefits are the prevention of colon cancer, coronary heart

48 diseases, obesity, diabetes and gastro-intestinal diseases [12, 13]. Tigernut has a total
49 carbohydrate content of 47.9 – 75.88%, of which starch is 17.2-39.2% and imparts undesirable
50 sensory attributes such as achalky mouthfeel to the products[14].

51 Coconut milk can play a vital role in balancing the nutritional deficiency of our diet. Coconut
52 (*Cocos nucifera*L.) is the most extensively grown and used nut in the world, which belongs to
53 the palm family (*Arecaceae*). Besides, it is an important commercial crop in many tropical
54 countries, contributing significantly to their economies [15]. The microorganism used mainly in
55 yoghurt production are Lactic acid bacteria which are utilised in the fermentation process.
56 *Lactobacillus* have been shown to have an inhibitory role on pathogenic bacteria [16].

57 The goal of this research was to evaluate the biochemical characteristics of the various
58 microbial isolates, the optimal growth temperatures and the effect of the incubation time and
59 inoculum concentration on the taste and desirability of the yoghurt.

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61 **2. MATERIALS AND METHODS:**

62 **Preparation of Yoghurt Starter Culture**

63 The morphology and type of bacterial colonies were investigated via serial dilution and cell
64 count experiments on MRS agar. Gram staining and microscopic examination of the growth
65 indicated that 5 distinct colonies designated ANOL1-5 were observed on MRS agar plates from
66 triplicate plates (Table 2). Morphological characteristics revealed that the isolates were
67 predominantly smooth margined with raised elevation, gram positive and white to creamish-
68 white in colour .

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70 **Identification by Morphological and Biochemical Assessments**

71 Three commercial yoghurts (Fan, Farmfresh and Habib) were obtained, serially diluted and grown
72 on different media. Visually distinctive colonies were randomly selected and purified by repeat
73 streaking on anti-fungal supplemented MRS agar, Nutrient agar and Muller Hinton agar.
74 Colonies that grew on both agars were gram stained to check deviations from the standard in
75 accordance with the standard Gram staining procedure described by Tortora [17]. Biochemical
76 tests were carried out in accordance with standard methods described by Cheesbrough[18].
77 Distinct isolates were subjected to morphological identification aided by Gram staining with the
78 following parameters: colour, Shape, Elevation, Surface and Biochemical characterization were
79 performed according to known methods [19].

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81 **Acclimatization of the Bacterial Culture to Ferment the Plant Milk**

82 The biochemically confirmed pure isolates were engineered in the production of to produce the
83 plant yoghurt. The optimized milk blend with the ratio 0.167C, 0.667T, 0.167D was also used to
84 achieve the desired product. The bacteria isolates were pressured to ferment 100% of the plant
85 base milk by preparing a fortified blend of plant milk (tiger nut, dates, and coconut) with cow
86 milk at various ratios of 1:10 to 10:1. The batch container having the highest volume of plant
87 milk and the evidence of microbial activities for yoghurt formation were serially diluted. The
88 diluents 10^6 and 10^7 , after serial dilution, were pour plated and anaerobically incubated in sterile
89 MRS agar, distinct colony was sub-cultured and later grown in sterilized MRS broth, the broth
90 was centrifuged at 10,000rpm and harvested into sterile water. The pure culture was then used as
91 inoculum. Incubation temperature, inoculum concentration and incubation time were used as
92 process variables, and all varied as provided in the experimental design (Table1) in order to
93 produce various samples of yoghurt formulation.

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Sensory Evaluation of the Probiotic Yoghurt from the optimized milk blend

Sensory evaluation of prepared yoghurt blends was carried out in accordance with the method described by Everitt [20]. The 9-point hedonic rating test was used for the assessment, ranging from 1- dislike to 9- like extremely (9-Like Extremely, 8-Like Very Much, 7-Like Moderately, 6-Like Slightly, 5-Neither Like nor Dislike, 4-Dislike Slightly, 3-Dislike Moderately, 2-Dislike Very Much, 1-Dislike Extremely). The sensory qualities assessed were colour, sweetness, taste, aroma, appearance and overall acceptability.

Experimental design:

Response surface methodology (Facecentered composite design) was used to obtain an optimal ratio formulation of coconut,tigernut and dates extracts used in the production of yoghurt. The model generated is quadratic and significant (p<0.05). Other terms in the ANOVA that exhibited significant p-values include A (inoculum concentration), BC (interaction of incubation time and temperature), B² (square of incubation time and C² (square of incubation temperature). The P-value for lack of fit is not significant and AdjR² value (0.7567 or 75.67%) is high. Being a Response Surface Methodology (RSM) design, it would first assume a Quadratic polynomial model as presented in the equation below.

$$Y = \beta_0 + \sum_{i=1}^3 \beta_i X_i + \sum_{i=1}^3 \beta_{ii} X_i^2 + \sum_{i=1}^3 \sum_{j=i+1}^3 \beta_{ij} X_i X_j$$

Where Y is the response, β₀ constant, β_i the linear coefficient, β_{ii} the quadratic coefficient, β_{ij} the interaction coefficient, X_i and X_j are the independent variables. The model for taste was therefore fitted based on the terms that showed significant p-values from the analysis of variance. The taste of yoghurt was modelled as specified below bore the regression coefficient values for the terms:

$$\text{Taste} = \beta_0 + A + BC + BC^2 + C^2$$

$$\text{Taste} = 6.80 - 0.16A - 0.12BC + 0.23B^2 - 0.39C^2$$

Table 1: Design key with the code and actual values of the independent variable

Independent Variable	1	0	+1
A. Inoculum concentration (%)	0.05	1.02	2.0
B. Incubation duration (h)	14.0	16.0	18.0
C. Incubation temperature (°C)	37	41	45

3. RESULTS:

Table 2: Culture and morphological characteristics of Lactobacillus strains obtained on MRS Agar

Microbial Code	Colour	Gram Reaction	Elevation	Margin	Surface	Microscopy observation
ANOL 1	White	Positive	Raised	Smooth	Spreading	Rod with rounded clusters
ANOL 2	Cream	Positive	Raised	Smooth	Spreading	Rod, rounded, in chain
ANOL 3	White	Positive	Raised	Smooth	Spreading	Cocci, single, in pair
ANOL 4	Cream	Positive	Raised	Smooth	Spreading	Rod, single pair, chain

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ANOL 5 Cream Positive Raised Smooth Small beads Spherical chain

128 **Key:** ANOL 1: *Lactobacillus acidophilus*; ANOL 2: *Lactobacillus bulgaricus*; ANOL 3:
 129 *Lactobacillus casei*; ANOL 4: *Lactobacillus lactis*; ANOL 5: *Streptococcus thermophilus*

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131 **Biochemical Characterization of the Isolates**

132 Biochemical characterization used to confirm the type of organisms isolated, revealed that all
 133 isolates were citrate, coagulase, indole, oxidase, urease, methyl red, nitrate, arginine, galactose,
 134 mannitol and raffinose negative. However, sugar tests conducted showed lactose,
 135 glucose, maltose, fructose, and sucrose were positive. This means the organisms can utilize these
 136 sugars as carbon sources for metabolism. Our plant yoghurt contains sucrose and it is important
 137 to note that these isolates can thrive in the sample. Salt and Acid tolerance on the isolates shows
 138 they can survive in low pH and hence in yoghurt. One attribute of LABs as probiotics is their
 139 ability to survive in salt environment and this test confirmed further the species. The results are
 140 displayed in Table 3.

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142 **Bacteria Temperature Growth in Milk Profile**

143 The isolates were exposed to various temperatures to evaluate survival. At a low temperature of
 144 4 -10°C, no growth was seen. At 27°C, there was partial growth for all isolates. Growth was
 145 observed at 37°C. At 40°C and 45°C, Full growth was observed, while no growth was observed
 146 at a high temperature of 60°C. Most *Lactobacillus* exhibits thermophilic tendencies and all
 147 isolates thrived most at temperatures between 40°C and 45°C, as shown in Table 4.

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149 **Table 3: Biochemical characterization of microorganisms towards starter culture**
 150 **development**

Biochemical test	ANOL 1	ANOL 2	ANOL 3	ANOL 4	ANOL 5
Citrate	-	-	-	-	-
Coagulase	-	-	-	-	-
Indole	-	-	-	-	-
Oxidase	-	-	-	-	-
Urease	-	-	-	-	-
Methyl –Red (MR) Test	-	-	-	-	-
Motility test	-	-	-	-	-
Salt endurance	1-9%	1 – 10 %	1-5%	1-10%	1-9%
Acid endurance	-	>2	-	-	>2
Nitrate reduction	-	-	-	-	-
Casein decomposition	-	-	-	-	-
Arginine producing ammonia	-	-	-	-	-
Glucose gas production test	-	-	-	-	-
Glucose	+	+	+	+	+
Galactose	-	-	-	-	-
Mannitol	-	-	-	-	-
Lactose	+	+	+	+	+
Maltose	+	+	+	+	+
Raffinose	-	-	+	-	+
Fructose	+	+	+	+	+
Sucrose	+	+	+	+	+
Trehalose	-	+	+	-	+

Organism	<i>Lactobacillus acidophilus</i>	<i>Lactobacillus bulgaricus</i>	<i>Lactobacillus casei</i>	<i>Lactobacillus lactis</i>	<i>Streptococcus thermophilus</i>
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151 **Key:** - Negative + Positive

152 ANOL 1: *Lactobacillus acidophilus*; ANOL 2: *Lactobacillus bulgaricus*; ANOL 3: *Lactobacillus casei*;

153 ANOL 4: *Lactobacillus lactis*; ANOL 5: *Streptococcus thermophilus*

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155 **Table 4.: Bacteria Temperature Growth in Milk from Tigernuts, Coconuts and Dates**

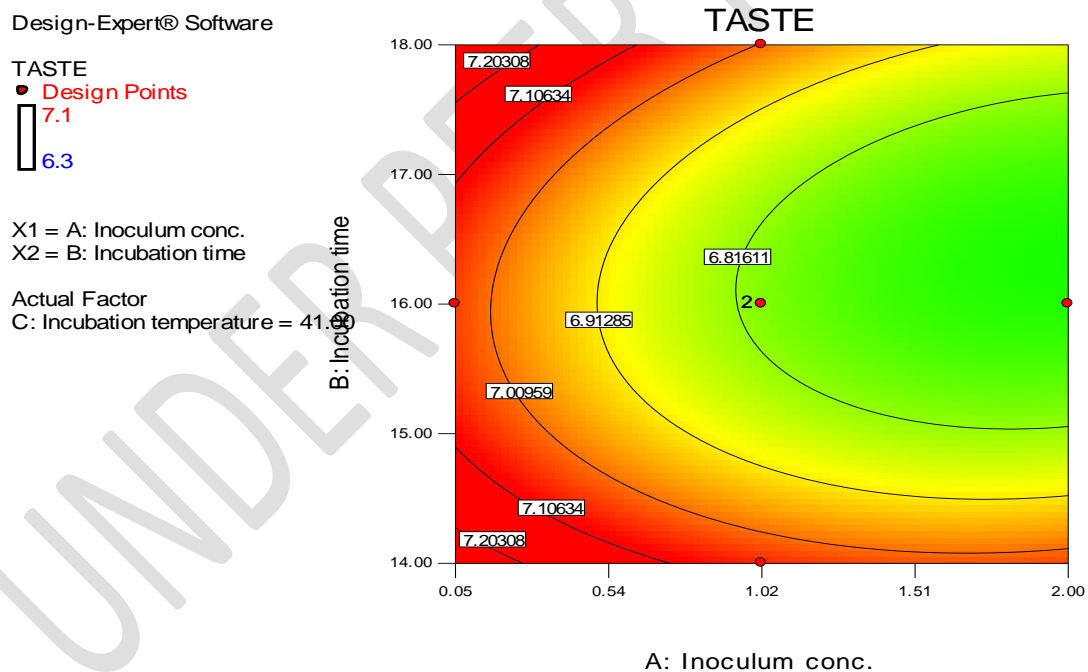
Isolates	Temperature					
	4°C	27°C	37°C	40°C	45°C	60°C
<i>Lactobacillus bulgaricus</i>	NG	PG	G	FG	FG	NG
<i>Lactobacillus acidophilus</i>	NG	PG	G	FG	FG	NG
<i>Streptococcus thermophilus</i>	NG	PG	G	FG	FG	NG
<i>Lactobacillus lactis</i>	NG	PG	G	FG	FG	NG
<i>Lactobacillus casei</i>	NG	PG	G	FG	FG	NG

156 **Key:** No Growth (NG) Growth (G) Partial Growth (PG) Full Growth (FG)

157

158 Figure 1 showed the interaction effect of inoculum concentration and incubation duration on the
 159 taste of the probiotic yoghurt. From the plot, the interaction between duration and inoculum
 160 concentration shows that the taste of the yoghurt improved from 7.1 to 7.2 when duration
 161 changed from 17hrs to 17.5hrs and inoculum concentration changed from about 0.7% to 0.4%.

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164 **Figure 1: Inoculum concentration and incubation time**

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166 Fig 2 shows the contour plot on taste when there is an interaction between incubation
 167 temperature and inoculum concentration. When the temperature changed from 44.5°C to 43.5°C
 168 and inoculum concentration changed from 1.02% to 0.45%, taste improved from 6.51 to 6.64.

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Design-Expert® Software

TASTE

● Design Points

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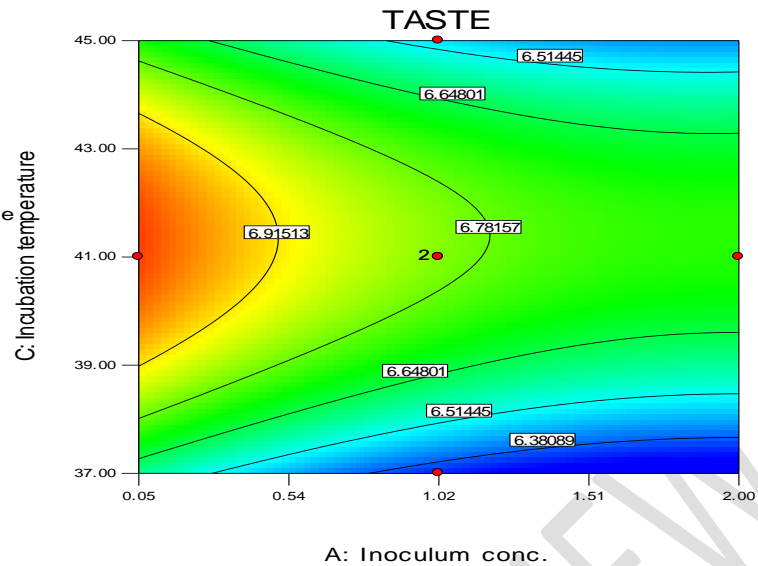
6.3

X1 = A: Inoculum conc.

X2 = C: Incubation temperature

Actual Factor

B: Incubation time = 16.00



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Figure 2: Contour plot for Inoculum conc and Incubation temp.

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Fig 3 shows the interaction between the Incubation temperature and the incubation duration on the taste of the probiotic yoghurt. From the contour, the taste of the yoghurt improved from 6.58 to 6.71 when the incubation temperature changed from about 45°C to 44°C and duration of incubation changed from 15.4hrs to 14.7hrs. There were 15 solutions that gave desirability of 1.0. Desirability ranges between 0.1 to 1.0, and the higher the desirability value the more the predicted value of the response variable gets closer to its actual (experimental) value. As shown in Fig.3, desirability increased from 0.379 to 0.804 when inoculum concentration changed from 2.0% to 0.54% and time changed from 14.3hrs to 15hrs. Incubation temperature was kept at 45°C.

Design-Expert® Software

Desirability

● Design Points

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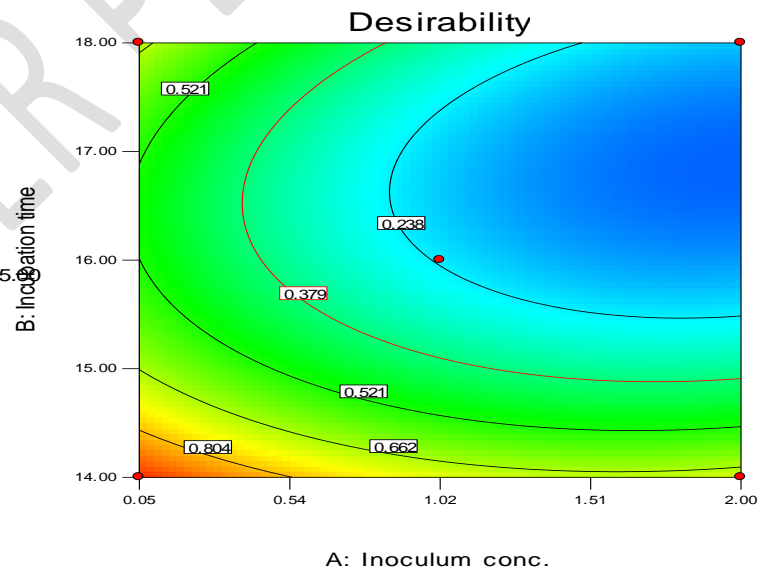
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X1 = A: Inoculum conc.

X2 = B: Incubation time

Actual Factor

C: Incubation temperature = 45.00



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Fig 3: Contour plot for Desirability of Taste

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Results of Syneresis are shown in Table 5. The syneresis result of the cow yoghurt (CY) and plant yoghurt (PY) over a 21 days period at different times (30, 60 and 90 minutes) showed that at 30 mins, cow milk yoghurt had values reducing from 12.85 to 10.00, while plant yoghurt reduced from 16.60 in day one to 15.56 at day twenty-one. At 60 mins interval, cow milk

190 yoghurt reduced from 16.60 in day one to 15.56 at day twenty-one, while plant yoghurt
 191 decreased from 20.00 to 17.00 respectively. At 90 mins, cow milk yoghurt syneresis reduced
 192 from 18.00 to 14.95, while plant yoghurt reduced from 24.75 to 21.05 for day one and day
 193 twenty-one respectively.

194 Result also showed that syneresis of the different yoghurt samples increased under a given
 195 storage condition as the time increased. Cow milk yoghurt stored at day one increased with time
 196 from 12.85 in 30mins to 18.00 after 90mins. Syneresis was seen to decrease throughout the
 197 storage period. Syneresis which is also termed “Wheying off”, was more in plant yoghurt than in
 198 cow yoghurt.

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Table 5: Syneresis (%) of the Plant and Cow yogurt.

Time Minutes	Sample	Strong Period Days			
		1	7	14	21
30	CY	12.85	12.00	10.75	10.00
	PY	16.60	16.60	16.60	15.65
60	CY	15.80	15.00	13.00	12.65
	PY	20.00	18.45	18.00	17.00
90	CY	18.00	17.85	15.50	14.95
	PY	24.75	23.45	23.00	21.05

201 **CY- Cow yoghurt; PY- Plant yoghurt.**

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3. DISCUSSION:

206 The combination of tigernuts, coconuts and date fruits can be said to have increased the aesthetic
 207 and sensory value of the yoghurt produced from the blend and this is in agreement with previous
 208 report [21]. The colours of samples 3, 6 and 8 were liked very much by the panelists while all
 209 other samples were liked moderately.

210 In this study, Taste and Overall acceptability were significantly different ($P < 0.05$) in their
 211 sensory scores. This is in agreement with findings from other studies, which revealed that
 212 incubation duration, incubation temperature, and starter culture concentration are factors to
 213 consider in plant-based yoghurt production [22, 23]. 2% inoculum as well as incubation
 214 temperature of about 41°C yielded the most acceptable (8.65 in sample 8) yoghurt as scored by
 215 the panelists. Lower concentrations of inoculum (0.05%) produced yoghurts that were slightly
 216 liked regardless of temperature and time. The process variables (Inoculum concentration,
 217 Incubation time and Incubation temperature) did not have and significant difference in the
 218 colour, sweetness, aroma and appearance of the sixteen (16) yoghurt samples. Whey separation
 219 (syneresis), also generally known as "wheying off" is a problem of fermented milks during
 220 storage and can be explained as the oozing off of liquid from the surface of a gel [24]. It
 221 negatively affects consumer perception and makes them believe the milk is microbiologically
 222 deteriorating. This study revealed that syneresis was higher in the plant yoghurt than the animal
 223 yoghurt. A number of factors influence yoghurt's viscosity, namely, milk composition, heat
 224 treatment, standardization method, selection of microbial culture, inoculum concentration,
 225 temperature, and fermentation duration [25]. The viscosity trend seen in yoghurt can be
 226 described as the interaction of the fiber and protein with the water of yoghurt, this forms a strong
 227 network that increases the resistance of yoghurt to flow. Stirred yoghurt has a peculiar
 228 characteristic, different from set yoghurt as its three-dimensional gel matrix is no longer visible.
 229 Stirred yoghurt has a weak gel system comprising of weakly associated clusters of proteins that

230 make up the network. During storage, stirred yoghurt exhibits thixotropic behavior and the
231 viscosity increased in both the plant yoghurt and the dairy yoghurt. In this study, the viscosity
232 increased significantly ($P>0.05$) and syneresis reduced during storage. This behavior is contrary
233 to what is seen in set yoghurt, where viscosity decreases with storage. This can be attributed to
234 increased lactic acid production as storage progressed, resulting in a more stable gel structure
235 [26]. The use of stabilizers, such as pectin, gelatin and starch, to try to prevent wheying-off is
236 strongly recommended, although another common approach is to increase the total solids content
237 of yoghurt milk.

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240 5. CONCLUSION:

242 This study revealed that incubation duration, incubation temperature, and starter culture
243 concentration are factors to consider in plant-based yoghurt production. The viscosity trend seen
244 in yoghurt can be described as the interaction of the fiber and protein with the water of yoghurt,
245 this forms a strong network that increases the resistance of yoghurt to flow.

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