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Assessment of the Impact of Temperature and Shelf Life on the Microbiological Quality of Feed Supplements Enriched with Probiotic Bacteria

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ABSTRACT

This study was initiated to assess the impact of shelf life on the microbiological quality and the influence of temperature on the vitality of probiotic strains contained in feed supplements made from whey. Three groups of samples were analyzed: Group I (A_3, C, TB), Group II (A_3, C, TB), and Group III (A_3, C, TB). Samples A_3, C and TB were stabilized feed supplements made from white sorghum flour successively enriched with probiotic strains of *Lactobacillus spp.* thermophilic, *Lactobacillus spp.* mesophile and *Bifidobacterium spp.* mesophile isolated from raw milk and whey whose initial concentrations were respectively 200.10^8, $250.10^{8,}$ and 80.10^7 CFU/g. Each complementary weighed 50g, and the moisture and dry matter contents were: 19.12% and 80.88%. Storage temperatures were between 2 and 4°C, 22 and 24°C and then 29 and 31°C for 42 days. The samples of groups I, II, and III were used to assess the influence of temperature on the vitality of the probiotic strains while group I was also used to evaluate the microbiological quality. The mass inoculation and surface spreading methods were used. The presence of germs indicating the state of hygiene, organoleptic quality and food poisoning was sought. Codex Alimentarius Commission and AFSSA 2007-SA-0174 standards were used in the interpretation of the results. The analysis results revealed the total absence of these germs. The appropriate temperature for storing probiotic strains is 2 to 4°C. In compliance with Codex Alimentarius Commission and AFSSA 2007-SA-0174 standards, the probiotic feed supplements analyzed are suitable for animal consumption.

Key words: Probiotics, Bacteria, Feed supplements, Shelf life and temperatures.

INTRODUCTION

The lack or absence of hygiene promotes the presence of microbes and other non-microbial agents in foodstuffs, causing cases of collective food poisoning threatening consumer health (Al-Humam 2019; Nguyen and Nguyen 2022). However, the main role of the diet is to provide nutrients to meet the physiological needs of the host. Consequently, a healthy and balanced diet is necessary to ensure this vital role. Indeed, many cases of "food poisoning" are notified each year; these incidents, in addition to the risk they represent for public health, often give bad publicity to the company (Delmas et al. 2006; Gauthaman 2023). The agri-food industry uses strains of bacteria in the manufacture of several food products. They are frequently positively associated with animal and human food, through the fermentation of a wide variety of products (Matamoros 2008; Aviles et al. 2020; Okonkwo and Igwilo 2022; Kalita et al. 2023). They are also present as technological flora in dairy, meat and plant products, bread-making yeasts and alcoholic beverages (Leroy and De Vuyst 2004; Vera-Santander et al. 2023). In recent years, the development and production of new foods containing probiotic microorganisms have attracted considerable interest due to their healthy properties (Kourkoutas et al. 2005; Rivera-Espinoza and Gallardo-Navarro 2010; Glago et al. 2021; Coniglio et al. 2023). Moreover, the incorporation of these probiotic bacteria as feed supplements in various dairy products has reinforced the acclaimed properties for health and given rise to an increasingly important consumption of these products. However, many locally produced products suffer from real stability problems and quickly become non-compliant from the point of view of microbiological and physicochemical quality (Rashid et al. 2023; Gul and Alsayeqh 2023).

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During production, they are exposed to biological contaminants such as undesirable microorganisms, parasites, viruses, mycotoxins, prions, and biotoxins at all stages of the production chain. This raises doubts among consumers who seem to attribute semi-finished or finished products as unsuitable characters. Therefore, the public authorities and FAO/WHO (2005) agree that healthy foodstuffs should be put on the market in order to protect health and ensure the safety of consumers. This concern affects not only imports and exports but also foods produced for local consumption (FAO/WHO 2005). According to Bousbia et al. (2018), the multiple food crises, the "mad cow" crisis, the "dioxin chicken" crisis, the melamine adulterated milk scandal (China), and numerous cases of food poisoning ended up instilling “food fears” among consumers. Thus, all this alarming information imposes vigilance on the food chain. To remove this ambiguity, the requirements of international institutions require manufacturers and food producers to do everything possible to ensure the sanitary and marketable quality of foodstuffs put up for sale in places of exchange (markets, shops, kiosks, etc.) (WHO 2012). This requirement is one of the greatest challenges for food science researchers today in order to gain consumer confidence. They must also pay particular attention to the stability of the products produced, which allows them to be stored for a long time while preserving their hygienic and organoleptic quality. So, the objective of this study is to evaluate the impact of shelf life on the microbiological quality and the influence of the temperature on the vitality of the probiotic strains contained in the feed supplements locally elaborated from whey.

MATERIALS AND METHODS

Study Framework

Laboratory of Microbiology and Quality Control of Foodstuffs (LAMICODA) of the Higher School of Biological and Food Techniques (ESTBA) of the University of Lome, Lome Togo served as a framework for the performance of microbiological analyzes.

Experimental Device

In total, three groups or types of samples were analyzed. Each group contained three types of samples. These were: group I (A_3, C, TB), group II (A_3, C, TB) and group III (A_3, C, TB). Samples A_3, C and TB were stabilized food supplements made from white sorghum flour successively enriched with probiotic strains: *Lactobacillus spp.* thermophilic, *Lactobacillus spp.* mesophile and *Bifidobacterium spp.* mesophile isolated from raw milk and whey whose initial concentrations were respectively 200.10^8, 250.10^8 and 80.10^7 CFU/g. Each complementary weighed 50 g and the water and dry matter contents were: 19.12% and 80.88%.

The samples from the three groups were all stored for 42 days. Only the storage temperatures were different from one group to another. Samples from group I (A_3, C, TB) were stored between 2 and 4°C, those from group II (A_3, C, TB) were stored between 22 and 24°C, then those from group III (A_3, C, TB) were stored between 29 and 31°C (Table 1). These feed supplements were formulated and stabilized on the basis of white sorghum flour (50g) whose moisture and dry matter contents were: 19.12 and 80.88% respectively. The choice of this duration and temperatures was inspired by the research work of Kailasapathy (2006), Georgieva et al. (2009), and Chun et al. (2014). During the analyses, the samples of group I, II, and III were used as part of the evaluation of the influence of temperature on the vitality of the probiotic strains. Contrary to groups II and III, the samples of group I were also used in the evaluation of microbiological quality in the feed supplements produced.

Table 1: Description of sample groups during experimentation

Sample groups	Probiotic supplements	Feed Storage (days)	Period Temperature (°C)
I	A_3, C and TB		2 to 4
II	A_3, C and TB	42	22 to 24
III	A_3, C and TB		29 to 31

A_3: *Lactobacillus spp.* Thermophilic, C: *Lactobacillus spp.* Mesophile and TB: *Bifidobacterium spp.* Mesophile

Microbiological Analysis

Three (03) groups of germs were taken into account in the analyses. These were hygiene indicator germs (Total Coliforms (CT), Thermotolerant Coliforms (Cth) or Fecal Coliforms (CF)); germs indicative of organoleptic quality (yeasts, molds) and indicator germs of food-borne illnesses (*Salmonella spp., Escherichia coli, Staphylococcus aureus*, and fecal Streptococci). The final concentration (after storage) in CFU/g of the germs making up the technological flora of the probiotic feed supplements was determined. These probiotic strains were: *Lactobacillus spp.* thermophilic, *Lactobacillus spp.* mesophile and *Bifidobacterium spp.* mesophilic.

Microbiological Analysis Methods

Preparation of Culture Media

The culture media were prepared according to the manufacturer's instructions. To a given mass of powdered culture medium, a precise volume of distilled water was added. Everything was brought to a boil until the powder was completely dissolved. The preparation obtained was distributed in sterile flasks or tubes to be sterilized in an autoclave at 121°C for 15min. The enumeration of the microbial flora associated with the stored probiotic feed supplements was carried out using the methods of inoculation in depth (mass) and surface spreading and by making the standard dilution: preparation of the stock solution. This involved a test sample of 25g of material to be analyzed (sample) in 225mL of Buffered Peptone Water (EPT) (CM0509), the whole incubated at 37°C for 24h (Guiraud 2003). Decimal dilutions ranging from 10^{-1} to 10^{-10} were prepared from this stock solution and then inoculated into culture media in Petri dishes. All these analyzes were carried out in a sterile atmosphere. The manipulations were carried out within a radius of fifteen centimeters from the flame of the Bunsen burner.

Enumeration of Microorganisms

Hygiene Indicator Germs

• Total coliform (CT) and Thermotolerant coliform (Cth): NF EN ISO 4832 (2006)

The enumeration of total and thermotolerant coliforms was done according to standard NF ISO 4832 (2006) which recommended the use of VRBL agar (TN1149). Petri dishes were incubated at 30°C for 24h (for total coliforms) and 44°C for thermotolerant coliforms (Table 2).

Organoleptic Quality Indicator Germs (fungal flora)

• Yeasts (L) and molds (M): NF EN ISO 21527-1 (2008)

The enumeration of yeasts and molds was carried out according to standard NF ISO 21527-1 (2008) on Sabouraud Chloramphenicol medium (BK027 HA) after incubation at 30°C for 3-5 days (Table 2).

Food Poisoning Indicator Germs

• Sulphite-reducing anaerobes (ASR) (Clostridia): NF EN ISO 15213-1 (2023)

Enumeration of Clostridia was done according to standard NF EN ISO 15213-1 (2023)
at 44°C for 48 hours on Tryptone Sulfite Neomycin (T.S.N) agar (BK001 HA) (Table 2).

• *Salmonella spp.* : NF EN ISO 6579 (2017)

The qualitative detection of *Salmonella spp*. was performed by pre-enrichment in buffered peptone water (37°C; 24h) and selective enrichment (37°C; 24h) in Rappaport-Vassiliadis broth (CM 0866, OXOID). After discoloration from Rappaport-Vassiliadis broth, cultures were isolated on Hektoen agar (CM 0419 OXOID) and Salmonella-Shigella agar (CM 0099 OXOID) and were then incubated at 37°C for 24h. The characteristic colonies were then cultured on Kligler-Hajna agar (DM 137 D). The characteristic colonies were used to carry out the confirmatory tests. They were Urea-Indole tests, the "Remel Rapid One System" gallery and serological tests for *Salmonella* NF EN ISO 6579 (2008) (Table 2).

• Staphylococci (*Staphylococcus aureus*): NF EN ISO 6888-3 (2003)

The enumeration of Staphylococci was carried out according to standard NF EN ISO 6888-3 (2003) at 37°C for 48 h. A volume of 0.1mL of dilution was spread on the surface using a spreader in a sterile Petri dish previously containing Baird-Parker (BP) agar (CM1127 OXOID) enriched with egg yolk and potassium tellurite (Potassium tellurite + mixed egg yolk; 5% of this mixture was added to 100mL of Baird-Parker). After incubation, black colonies with or without a clear halo were used to search for free staphylocoagulase (agglutination test with staphytect plus: Staphytect reagent + one colony, which could indicate a positive or negative reaction) (Table 2).

• Search and enumeration of fecal streptococci

Group D streptococci or fecal streptococci are sought and counted on the selective agar medium. From the decimal dilutions, a volume is carried aseptically in a Petri dish containing Slanetz and Bartley agar (CM0377 OXOID). The dishes thus inoculated were incubated at 37°C for 48h. The characteristic enterococci colonies are pink-red to brown. Colony identification is confirmed by subculture on Litsky medium at 37°C for 24h (Guiraud 2003; Elmarkhi et al. 2017**)** (Table 2).

• *Escherichia coli*: NF ISO 16649

TBX Agar is a selective medium for the enumeration of β-D-glucuronidase positive *E. coli* in food products and samples from the production environment. The result is obtained directly by counting the characteristic colonies after only 24h of incubation, without it being necessary to carry out a confirmation step. Characteristic colonies show blue to blue-green colonies. The standard formula meets the composition defined in the standards NF ISO 16649-1 (2018), NF EN ISO 16649-3 (2015) and NF ISO 16649-2 **(**2001) (Table 2).

Determination of the Final Concentration of Germs Making up the Technological Flora of Preserved Probiotic Feed Supplements

The technological flora of probiotic feed supplements was composed of three (03) germs of interest which were: *Lactobacillus spp.* mesophilic, *Lactobacillus spp.* thermophilic and *Bifidobacterium spp.* mesophile. In order to determine the concentration of these germs after storage, they were counted on agar media: MRS (CM 1153 OXOID) and MRS modified by the addition of 0.05% L-Cysteine hydrochloride. The preparation of dilutions of these feed supplements consisted in preparing the stock solution by diluting 10g in 90mL of Tryptone-Salt broth (TS), followed by homogenization for 3 min. The preparation was decanted for 30 min which corresponded to the necessary revivification time of the germs sought.

Table 2: Summary of the analysis methods used when looking for germs related to studies of the microbiological quality of stored probiotic feed supplements

Germs	Culture media	Seeded volume	Inoculation methods	Incubation		Methods reference
				Temp (°C)	Time (hours)	
CT	VRBL	1mL	EM	30	24	NF EN
CTH	VRBL	1mL	EM	44	24	ISO 4832 (2006)
Organoleptic quality indicator germs						
L	SAB	1mL	EM	30	48	NF EN
M	SAB	1mL	EM	30	48	ISO 21527-1 (2008)
Food poisoning indicator germs						
Sal-monella spp.	Ra-V	0.1mL	EM	37	24	NF EN ISO 6579, (2017)
S. aureus	Baird-Parker	0.1mL	ES	37	48	NF EN ISO 6888-3 (2003)
Fecal streptococci	S & B	1mL	EM	37	48	
ASR	TSN	1mL	EM	44	48	NF EN ISO 15213-1 (2023)
E. coli	TBX Agar	1mL	EM	44	24	NF EN ISO 16649-2 (2001)

ACT: Total coliforms, CTH: Thermotolerant coliforms, L: Yeasts, M: Molds, *S. aureus*: *Staphylococcus aureus*, ASR: Sulphite-reducing anaerobes, *E. coli*: *Escherichia coli*, EM: Inoculation into the Mass, ES: Surface spreading, VRBL: Violet Red Bile Lactose Agar, S & B: Salnetz and Bartley Medium, SAB: Sabouraud Chloramphenicol Agar and Ra-V: Rappaport-Vassiliadis, TSN: Tryptone Sulfite Neomycin Ager and Temp Temperature.

Finally, the solution obtained was used to prepare decimal dilutions by successively adding 1mL of the previous solution to 9mL of sterile Tryptone-Salt (TS) until a dilution of 10^{-10} was obtained (Table 3).

Results Determination

Counting was carried out according to the NF EN ISO 7218 standard updated in October 2007.

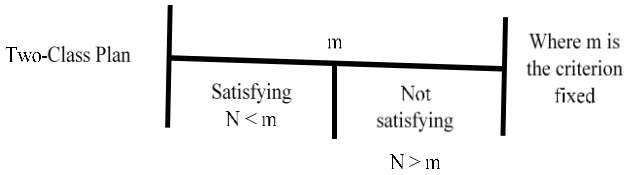
This involves counting all the colonies that have grown on the boxes, taking the following factors into account:

- Only count the boxes containing between 10 and 300 colonies,
- Always multiply the found number by the inverse of its dilution,
- Then calculate the arithmetic mean of the colonies between the various dilutions.
- The results obtained, expressed in colony format units (CFU) per dish, are then taken up in CFU/g by applying the formula:

$$N = (\sum \text{Colonies})/(\text{VmL} \times (n1 + 0.1\, n2) \times D)$$

Where:

Σ Colonies: sum of the numbers of bacterial colonies in the Petri dish considered; N: number of CFUs per g of initial product; VmL: Inoculated volume in mL; n1 and n2: number of interpretable dishes chosen at the 1st and the 2nd dilution considered and D: dilution factor of the first dilution considered. A two-class interpretation plan was used for the determination of the quality of the samples tested following the microbiological criteria applicable to ready meals in commerce defined by the standards developed by the Codex Alimentarius Commission (CAC), (2004); (2007) and AFSSA 2007- SA-0174 (2008).



RESULTS

Microbiological Analysis Results

Table 4 presents the analysis records of the different germs sought in the A3, C and TB samples of group I, all were stored at 2 to 4°C for 42 days.

The microbiological analyzes carried out on the samples A_3, C, and TB of group I revealed that the germs: total coliforms, thermotolerant, the fungal flora (yeasts and molds), sulfite-reducing anaerobes (Clostridia), *Escherichia coli*, fecal streptococci, *Salmonella spp.*, and *Staphylococcus aureus* were all absent in these samples (<10).

Influence of Temperature on the Vitality of Probiotic Strains and Feed Supplement Labeling

Table 5 shows the labeling and the influence of temperature on the final concentration of the germs making up the technological flora of probiotic feed supplements.

The viability of *Lactobacillus spp.* thermophilic (A_3), *Lactobacillus spp.* mesophilic (C) and *Bifidobacillus spp.* mesophilic acid (TB) was evaluated for a storage period of 42 days at different temperatures: 2 and 4°C, 22 and 24°C then 29 and 31°C in groups I, II, and III. The results obtained showed that the temperature considerably reduced the final microbial concentration in CFU/g in the samples analyzed after their storage.

Table 3: Final concentration of germs making up the technological flora of probiotic feed supplements before storage.

Microbial groups	Culture media	Seeded volume	Inoculation methods	Incubation		Microbial load at the start of storage
				Temp (°C)	Time (hours)	
Lactobacillus spp. mesophilic	MRS	1mL	EM	37	72	250.10^8 CFU/g
Lactobacillus spp. Thermophilic	MRS + L-Cysteine hydrochloride	1mL	EM	37	72	200.10^8 CFU/g
Bifidobacterium spp. mesophilic	MRS + L-Cysteine hydrochloride	1mL	EM	37	72	80.10^7 CFU/g

Abbreviations: MRS: deMan Rogosa Charpe, EM: Inoculation into the Mass and Temp: Temperature.

Table 4: Overall overview of the results of the count of germs indicating hygiene and organoleptic quality with their microbiological criteria

N°	Germs involved	Number of germs in UFC/g	Microbiological criteria	Conformity	Methods reference
1	Total coliforms	<10	10^3 CFU/g		NF EN ISO
2	Thermotolerant coliforms (CTh	<10	10 CFU/g	Compliant	4832 (2006)
		Organoleptic quality indicator germs			
3	Yeasts	<10	10^3 CFU/g	Compliant	NF EN ISO
4	Molds	<10	10^3 CFU/g		21527-1 (2008)
		Food poisoning indicator germs			
	Germs involved	Number of germs in CFU/g	Target value	Tolerance value	Methods reference
5	*Escherichia coli*	<10	<10	<100	NF EN ISO 16649-2 (2001)
6	Sulfito-reducing anaerobes (Clostridia)	<10	<10	<100	NF EN ISO 15213-1 (2023)
7	Fecal streptococci	<10	<10	<100	
8	*Salmonella sp.*	<10	Absent/25g	-	NF EN ISO 6579, (2017)
9	*Staphylococcus aureus*	<10	<10	<100	NF EN ISO 6888-3 (2003)

Presentation of results with microbiological criteria: Table 4 presents the results of the count of germs that are indicators of hygiene and organoleptic quality resulting from the analyzes with their microbiological criteria.

o Total coliforms

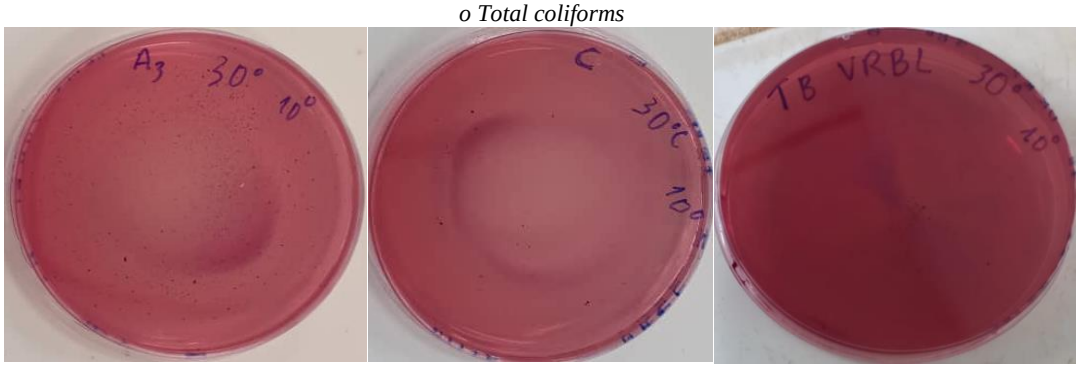


Fig. 1: Photograph showing the search for the presence of total coliform germs in samples A_3, C and TB: Germ diversity and trend from microbiological analyzes of samples A3, C and TB stored at 2 to 4°C for 42 days: Figures (1 to 8) present the results of the various germs sought: Hygiene indicator germs (total and thermotolerant coliforms): The obtained results showed the absence of total coliforms in the samples analyzed at the first dilution.

o *Thermotolerant coliforms*

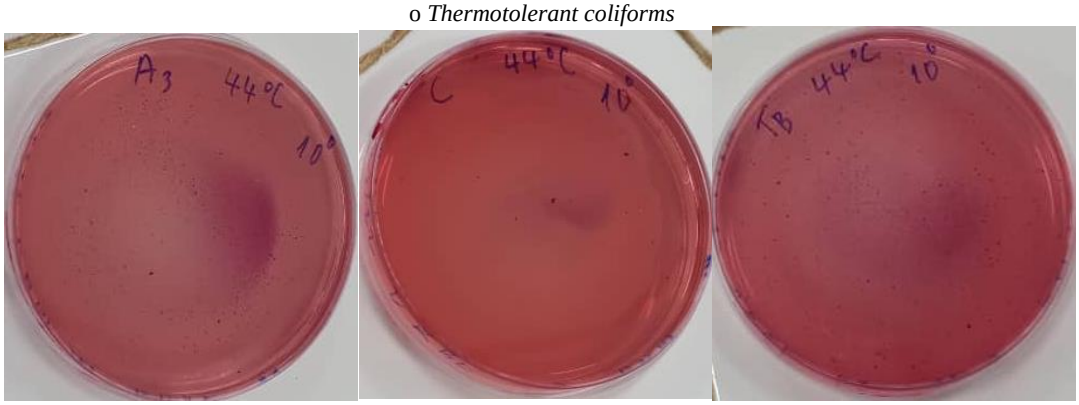


Fig. 2: Photograph showing the search for the presence of thermotolerant Coliform germs in samples A_3, C and TB: The results of the microbiological analyzes of these samples revealed the absence of thermotolerant coliforms in the samples at the first dilution.

o *Yeasts and Molds*

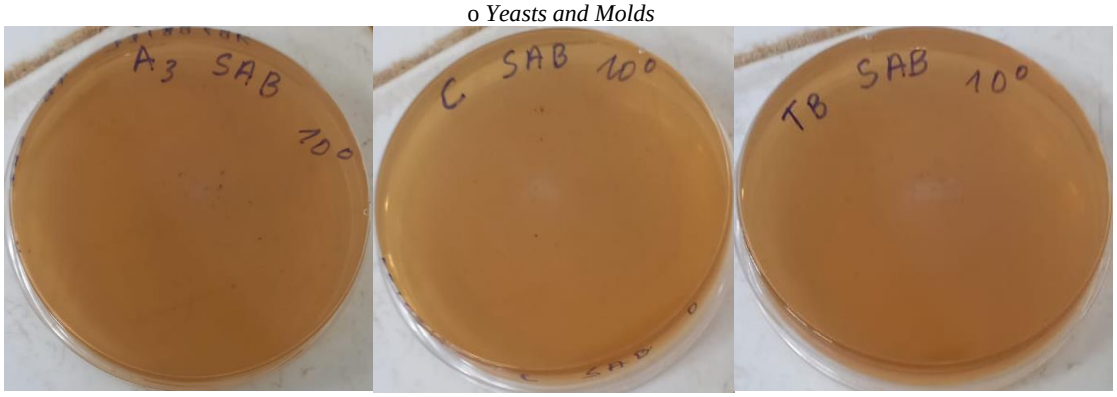


Fig. 3: Photograph showing the search for the presence of fungal flora (yeasts and moulds) in samples A_3, C and TB: The results of the microbiological analyzes of these samples revealed the absence of Yeasts and Molds in the samples at the first dilution: Organoleptic quality indicator germs (yeasts and molds).

Table 5: General information on feed supplements stored at different temperatures for 42 days under the "probiotic" label.

Groups	Feed supplements	Probiotic bacteria	Net weight (g)	T°C and shelf life	SCF to CFU/g
	A_3	*Lactobacillus spp.* Thermophilic	50	2 to 4°C for 42 days	185.10^8
	C	*Lactobacillus spp. mesophilic*			236.10^8
I	TB	*Bifidobacterium spp.* mesophilic			68.10^7
	A_3	*Lactobacillus spp.* Thermophilic	50	22 to 24°C for 42 days	120.10^5
	C	*Lactobacillus spp. mesophilic*			160.10^4
II	TB	*Bifidobacterium spp.* mesophilic			$17.\ 10^4$
	A_3	*Lactobacillus spp.* Thermophilic		29 to 31°C for 42 days	25.10^5
	C	*Lactobacillus spp.* mesophilic			45.10^4
III	TB	*Bifidobacterium spp.* mesophilic	50		10.10^4

Abbreviations*: a_w: moisture content, DM: dry matter content T: temperature, SCF: final microbial concentration after storage.*

o Clostridia (Sulphite-reducing anaerobes (SRA))

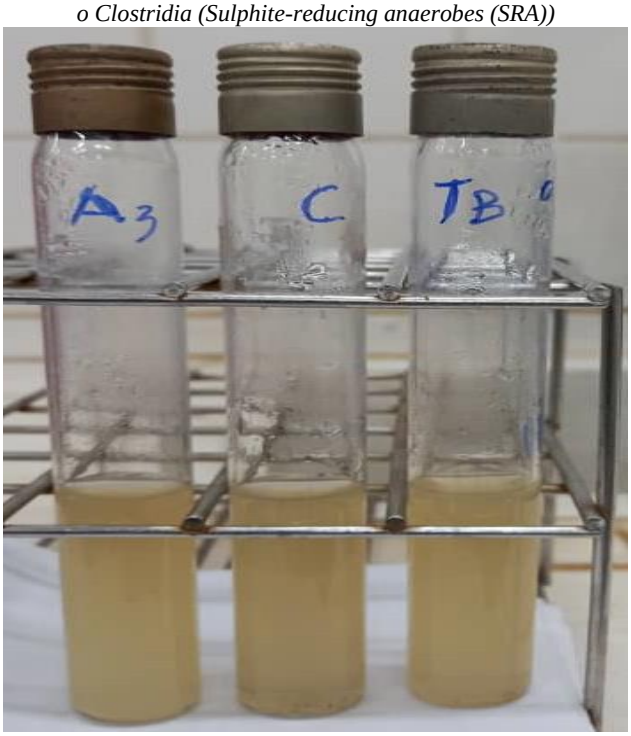


Fig. 4: Photograph showing the search for the presence of sulphite-reducing anaerobes (ASR) (Clostridia) germs in samples A_3, C and TB: The results from the microbiological analyzes of these samples revealed the absence of sulphite-reducing anaerobic bacteria (ASR) in the samples at the first dilution: Germs indicative of food poisoning (Clostridia, fecal streptococci, *E. coli*, *Salmonella spp*. and *Staphylococcus aureus*

DISCUSSION

The research results of the fungal flora revealed the non-existence of yeasts and molds in the A_3, C, and TB samples analyzed. This lack of fungal isolates made it possible to preserve these feed supplements for a long time without harming the microbiological quality which could be suspected as unfit for consumption (Fig. 3; Table 4). These results were in accordance with those found by Torkar and Teger (2008) who showed that yeasts and molds or microscopic fungi are not very virulent parasites and could alter the organoleptic qualities and lead to the accumulation of toxic secondary metabolites including mycotoxins. Among these are aflatoxins, ochratoxin A, fumonisins, trichothecenes, fusarins, zearalenone and ergot alkaloids which are known to be hepatotoxic, nephrotoxic, immunotoxic and carcinogenic (Kaushal and Sinha 1993; Ward et al. 2002; Abrar et al. 2013; Derntl et al. 2017). In addition, the existence of yeasts and molds (*Aspergillus, Penicillium, Mucor, Fusarium*) can compromise the long-term preservation of sorghum flour since their presence in a product exposes it to other types of microorganisms (Russell et al. 2017; Pérez-Lavalle et al. 2020).

The results obtained during the counting of total coliforms showed that there was an absence of these germs in the A_3, C, and TB samples analyzed (Fig. 1; Table 4). This absence was explained on the one hand by the absence of the fungal flora and on the other hand by the fact that the hygiene measures were applied in the stages of the production chain and conservation. These results were consistent with those found by El-Ziney and Al-Turky (2007) who showed that the absence of total coliforms is seen generally as an indicator of good hygienic practice during handling. The results of research on Thermotolerant Coliforms or Fecal Coliforms in feed supplements have made it possible to assess the hygiene conditions that prevailed during the development of these products. The absence of these germs in the samples of group I (A_3, C, and TB) analyzed proved that hygienic conditions were also required during the production and handling of these supplements (Fig. 2; Table 4). These results corroborated those found by Dogˇan-Halkman et al. (2003) who showed that the absence of fecal coliforms in the analyzed milk samples was synonymous with decontamination. Among the microorganisms sought in all of these samples, some are extra-intestinal pathogens.

The search result for *E. coli* in the A_3, C and TB samples showed that they were absent in these samples. These presumed pathogenic microorganisms extra-intestinal are the most common cause of urinary and blood infections in animals and humans (One Health approach) and are also reliable indicators of food contamination. Moreover, *E. coli* bacteremia's are responsible for the failure of prophylaxis, therapy, and metaphylaxis in animals and humans since this *E. coli* is an important reservoir for antimicrobial resistance genes and consequently leads to an extension of hospitalization period and increased mortality in hospitals (Naylor et al. 2019; Bonten et al. 2021; Leger et al. 2021). This noted absence testified to non-defective hygiene during the development of these feed supplements (Fig. 6; Table 4). This result was consistent with that found by Dogˇan-Halkman et al. (2003) who showed that the absence of *E. coli* in a sample depended on compliance with hygiene measures by the manufacturer and/or the immediate environment of the product. The search result for Staphylococci in the various A3, C, and TB samples analyzed revealed their total absence. This absence of *Staphylococcus aureus* in these samples showed that there was no contamination during the development of these feed supplements since *S. aureus* is a Gram-positive bacterium, a pathogen contaminating milk and dairy products causing food poisoning mainly due to its enterotoxins (Fig. 8; Table 5) (Gebremedhin et al. 2022). *S. aureus* is a bacterium commonly found in the nostrils, on the skin, and on the hair of warm-blooded animals, including humans. It can produce a wide variety of virulent factors, including staphylococcal enterotoxins (Grispoldia et al. 2021). Treatment of *S. aureus* infections is complicated by antibiotic resistance and no effective vaccine is available (Gordon et al. 2021). This result was consistent with that found by N'goran-aw et al. (2018) who proved that the absence of Staphylococci was a sign of good practice during production and handling since their presence in a product caused an alteration of microbiological quality and consequently a source of food poisoning. This result was also consistent with that found by Belhadj et al. (2004) who explained that the presence of staphylococci in a dietary supplement exposed consumers to a health risk. Similarly, *Salmonella* contamination is mainly associated with products such as poultry, livestock, and their feeds (Ehuwa et al. 2021). The *Salmonella* search result showed that no *Salmonella* was found in all the analyzed samples. The search for these Enterobacteriaceae in foodstuffs is

o Fecal streptococci

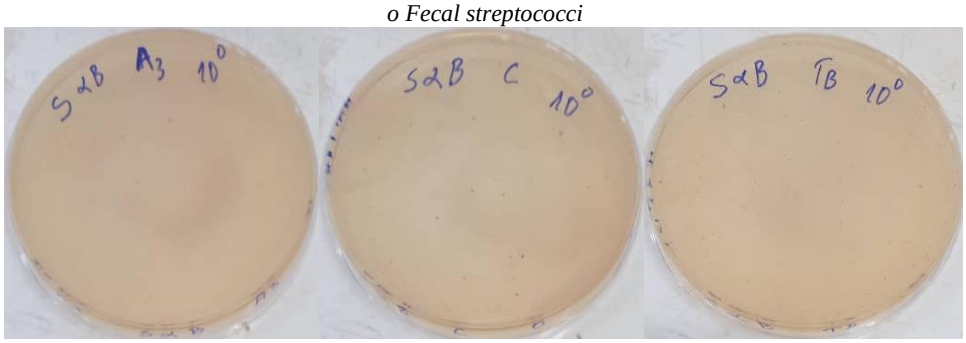


Fig. 5: Photograph showing the search for the presence of fecal Streptococci in samples A_3, C and TB: The results obtained from microbiological analyzes of these samples showed the absence of fecal Streptococci in the samples at the first dilution.

- *E. coli*

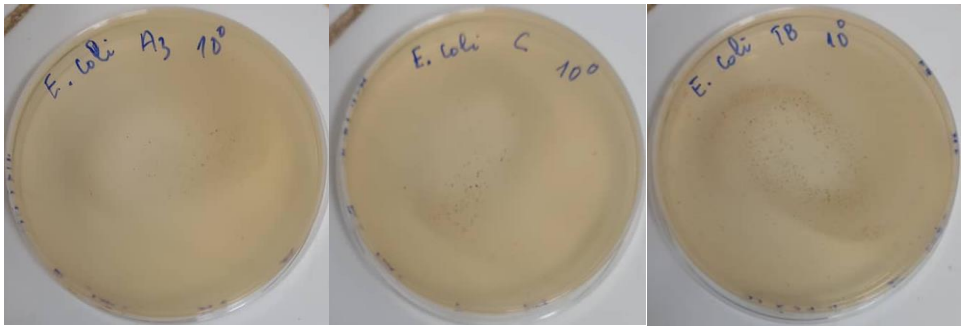
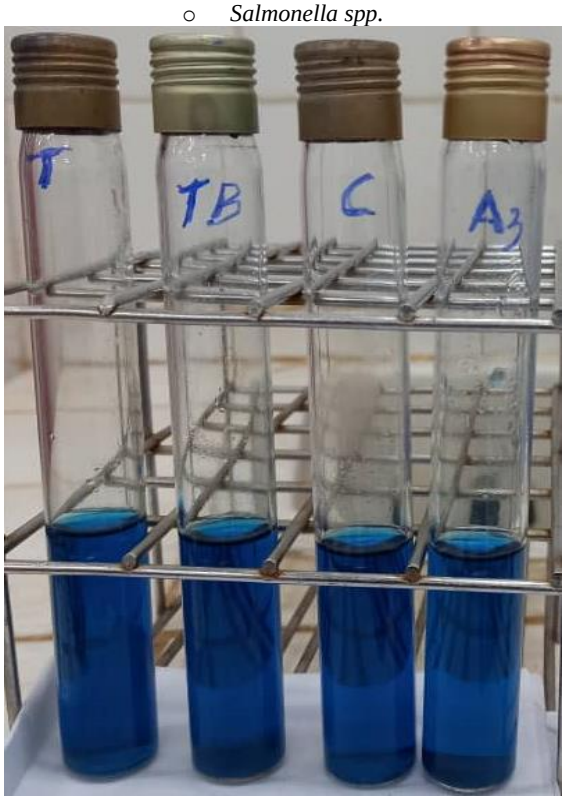


Fig. 6: Photograph showing the search for the presence of *E. coli* in samples A_3, C and TB. The results obtained from the microbiological analyzes of these samples showed the absence of *Escherichia coli* in the first dilution samples.

- *Salmonella spp.*



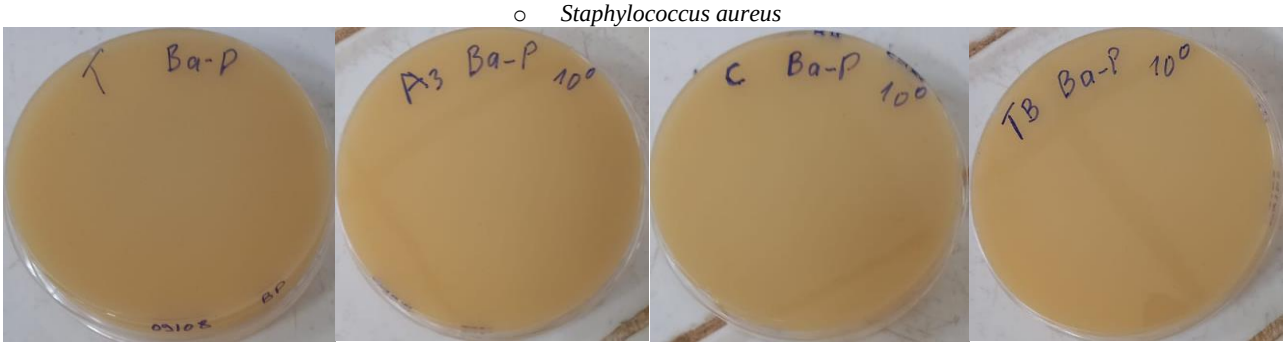
T: control sample

Fig. 7: Photograph showing the presence of *Salmonella spp.* in samples A_3, C and TB: The results obtained from the microbiological analyzes of these samples have shown that there is no discoloration of the Rappaport-Vadilliadis medium during enrichment. So, the process of finding *Salmonella spp.* stopped and this species was absent from the analyzed samples.

important because any product intended for animal and human consumption must not contain them (Fig. 7; Table 5). This result was in line with those found by Dennaï et al. (2001) who also proved the absence of *Salmonella spp.* in food intended for human consumption. The search result for Fecal Streptococci and Clostridia revealed a total absence of these germs in all the samples analyzed. This can be explained by the application of good hygiene practices during handling and production (Fig. 8; Table 8) since fecal coliforms and streptococci are good indicators of fecal contamination, and their presence is associated, in the majority of cases, with that of pathogenic germs and the research work of Lim et al. (2020) revealed that *Clostridium difficile* is a pathogen that contaminates food and the environment. The vitality of bacterial isolates during the storage period remains a very important property of cultures intended for use as probiotics.

A concentration of living cells of probiotic bacteria after storage has been set by international organizations. In fact, for the 42 days of storage between 2 and 4°C, the live bacterial cells counted were 185.10^8, 236.10^8 and 68.10^7 CFU/g respectively in the A_3, C and TB samples of group I. This result was consistent with that found by Kailasapathy (2006) who reported that the viability of probiotic cells remained relatively constant at 4°C for more than one month. On the other hand, storage at 22 to 24°C for 42 days significantly reduced the number of live cells by 120.10^5, 160.10^4 and 17.10^4 CFU/g respectively in A_3, C and TB samples of group II. This number of living cells was insufficient to exert a probiotic effect, since, for probiotic strains, FAO and WHO (2005) recommended a minimum of 10^6 CFU of viable probiotic bacteria per gram. Moreover, according to Talwalkar and Kailasapathy

- *Staphylococcus aureus*



T: Control Petri dish

Fig. 8: Photograph showing the presence of Staphylococci in samples A_3, C and TB. The results obtained from the microbiological analyzes of these samples showed that there was no *Staphylococcus aureus* in the samples analyzed at the first dilution.

(2004), minimum concentrations of 10^6 and 10^7 CFU/g in the finished product are considered therapeutic amounts of probiotic cultures in processed foods. This result was also in accordance with those found by Chun et al. (2014) who noticed a significant reduction in the concentration of *Lactobacillus plantarum* DKL 109 after storage at 25°C. Moreover, for storage between 29 and 31°C during the same period, the microbial concentration obtained was 25.10^5, 45.10^4 and 10.10^4 CFU/g respectively in the A_3, C and TB samples of group III. This result corroborated those found by Chun et al. (2014) who noted an 80% reduction in the concentration of *Lactobacillus plantarum* DKL 109 stored at 37°C in its free form. In general, the absence of germs indicating the state of hygiene organoleptic quality and food toxic infections in the samples A_3, C and TB of the group I analyzed is linked to good hygiene practice and complies with the technological process, in particular the sterilization of sorghum flour (121°C during 20min). This could also be due to the inhibiting role that lactic acid bacteria exert on the different flora through their metabolic products, namely lactic acid, which makes the environment hostile for most undesirable bacteria. Given the sterilization temperature of sorghum flour (121°C) and the absence of these germs in all the samples analyzed, the results obtained in this experiment were in accordance with Louis Pasteur's "Germ Theory". According to the postulate of Pasteur et al. (1878), spontaneous generation is not possible. This result was consistent with those found by Fayol-Messaoudi et al. (2005) and Macaluso et al. (2016) who showed that lactic acid bacteria played an important role in the reduction or elimination of the contamination flora, by the production of lactic acid and inhibiting substances. This absence could also be related to the low water activity (a_w = 19.12%) contained in these preserved samples, as was the case in the T45 and T65 flours. These results corroborated those found by Breton and Zwaenepoel (1991) and Couture (2000) showed that the content of moisture (a_w) below 20% was unfavorable to the emergence of fungal flora in flours during storage. Moreover, all the results obtained during the count were all less than ten (<10) (Tables 4 and 5) and analogically all less than "m". So, they complied with the microbiological standards developed by the Codex Alimentarius Commission (CAC), (2004); (2007) and AFSSA 2007-SA-0174 (2008), hence food supplements enriched with *Lactobacillus spp.* thermophilic, *Lactobacillus spp.* mesophile and *Bifidobacterium spp.* mesophiles stored between 2 and 4°C under the probiotic label analyzed were suitable for animal consumption.

Conclusion

The 42 days of storage between 2 and 4°C of feed supplements enriched with *Lactobacillus spp.* Thermophilic, *Lactobacillus spp.* mesophile and *Bifidobacterium spp.* mesophilic does not have a negative impact on the microbiological quality of Group I samples. The analysis showed no presence of total and thermotolerant coliforms indicating the hygienic state of the samples. No yeast or mold was found indicating the organoleptic quality of the samples. Besides, no toxic germs like *Escherichia coli,* Clostridia, Fecal streptococci, *Salmonella spp.* and *Staphylococcus aureus* causing infections were found. In the same way, the conservation of samples A3, C and TB of group I under the probiotic label between 2 and 4°C also has no negative impact on the vitality of these strains. On the other hand, storage between 22 and 24°C then 29 and 31°C considerably reduced the concentration in CFU/g of these strains contained in the feed supplements of groups II and III analyzed. The results obtained in the context of microbiological quality were all in compliance with the standards developed by the Codex Alimentarius Commission (CAC), (2004); (2007) and AFSSA 2007-SA-0174 and therefore the A3, C and TB group I feed supplements analyzed were all clean for animal consumption. Other studies are envisaged to incorporate these feed supplements in the diet of chickens.

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Conflict of interests: We, the authors of the accompanying article, write to declare that there is no personal or professional conflict of interest with our work.

Data Availability: The datasets generated during and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Author's Contribution: All authors contributed to the study's conception and design. The first draft of the manuscript was written by Glago Jean. All authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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