Assessment of the Potential of Lactic Acid Bacteria as Dried Starter Culture for Cereal Fermentation

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Abstract: The kinetic growth parameters [maximal specific growth rate μ_m (h^{-1}), and generation time *g* (h)] and viability after stress treatments (dehydration with glycerol solution of increasing concentration, freezing or heating stress) of four lactic acid bacteria [*Lactobacillus plantarum* (LP), *L. fermentum* (LF), *Leuconostoc* spp. (M1) and *Lactococcus* spp. (M2)] were determined. The maximal specific growth rate (μ_m) and generation time *g* ranged between 0.51 h^{-1} to 0.14 h^{-1} and 0.61 h to 1.33 h, respectively. We observed that the strains were sensitive to a change in water activity (a_w 0.32 final), storage and incubation temperatures. The Strain LF obtained a higher cell concentration and viability, as well as a lower *g* compared to those obtained by the other strains. The kinetic growth parameters (μ_m, g) together with viability after stress treatments could be used for the screening of dried lactic acid starter cultures.

Keywords: Dehydration tolerance, generation time, lactic acid bacteria, starter cultures, stress treatment, viability, water activity.

INTRODUCTION

Traditional fermented cereal based foods consumed in Africa are processed by natural fermentation of maize, sorghum and/or millet and are particularly important as weaning foods for infants and dietary staples for adults [1, 2]. The use of dried lactic acid bacteria (LAB) starter cultures during fermented cereal based foods processing could result in a high degree of control over the fermentation process and standardization of the end product. We need to take into account technological effectiveness when selecting strains for food fermentation. Commercial starter cultures are supplied in concentrated form by freeze-drying, vacuum-drying, spray-drying, drum-drying, fluidized bed drying or air-drying. These techniques impose environmental stress on the bacterial cells, such as freezing, drying, long-term exposure to low water activities and re-hydration. Moreover, during cereal fermentation processes, LAB are exposed to various environmental stress conditions, such as temperature fluctuations, acid, pH, high osmotic pressure and absence of available nutrients.

The suitability of LAB starter cultures for large-scale production and their stability during drying and storage in the dried state are important criteria for dried starter culture selection. The evaluation of cell viability under different stress conditions could be a valuable tool to evaluate the potential of lactic acid bacteria to be produced in the dried state [3]. The ability of LAB to establish them-selves and to dominate the LAB population during cereal dough fermentation is another important characteristic of a starter culture. The dominance of the starter culture would be exerted by its fast and predominant growth under fermentation conditions. In predictive food microbiology, the generation time (*g*) is often used to characterize bacterial growth curve [4]. The generation time is the time needed for doubling the initial population during the exponential growth phase. It is directly linked to the maximal specific growth rate (μ_m), which is the slope of the logarithm of the growth curve in the exponential growth phase.

The objective of the present study was to determine the kinetic growth parameters (μ_m, *g*) and viability under stress treatments in order to assess the ability to tolerate dehydration of LAB isolated from fermented cereal foods in Côte d'Ivoire.

MATERIAL AND METHODS

Microorganisms and Preparation of the Seed Bank

The strains *Lactobacillus plantarum* (LP), *L. fermentum* (LF), *Leuconostoc* spp (M1) and *Lactococcus* spp (M2), belonging to the collection of lactic acid bacteria of our University, were used. The strains had previously been isolated from cereal based fermented foods. Each strain was inoculated in MRS broth and incubated at 30°C for 18 h. The

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cells obtained after concentration (2500 ×g, 20 min) were maintained in 50% (v/v) glycerol and frozen at -80°C (stock culture). Fifty µl of the stock cultures were used to inoculate 150 ml of MRS broth, which was incubated at 30 ± 2°C for 22 h. Ten ml aliquots of the culture broths were withdrawn at 2h intervals during incubation, and used for growth determinations.

Analytical Methods

Viable counts were carried out on MRS agar medium with incubation at 30°C for 48h. Dry weight (DW) was obtained after drying at 105°C until constant weight was achieved. Viable counts were expressed on dry weight basis. The pH of the fermentation broths was determined using a pH-meter. The maximal specific growth rate (μ_m) and the generation time (g) were obtained by directly fitting the logarithmic transformation of plate counts versus time:

$$g = \frac{0.693}{\mu m} \quad (1)$$

$$\mu_m = \frac{\text{Ln } N_1 - \text{Ln } N_0}{t_1 - t_0} \quad (2)$$

Where g (h) is the generation time, N_0 the initial population at time t_0 (cfu/g DW), N_t is the population at time t (cfu/g DW), and μ_m (h^{-1}) is the maximal specific growth rate.

Assessment of Ability to Tolerate Dehydration

Effect of Dehydration with Glycerol Solution on Viability of Tested Bacteria

About fifty µl of each stock culture was inoculated in 150 ml MRS broth and incubated at 30°C for 18 h. The cell pellets obtained after concentration (2500×g, 20 min) were re-suspended in 1 ml 0.5 % peptone salt solution. One ml of 90% (w/w) glycerol was added to the cell suspension, homogenized and allowed to stand for 15 min at room temperature. The same procedure was repeated four times, and the cell suspension obtained was concentrated by centrifugation (2500×g, 3 min) followed by two washing steps with 0.5 % peptone salt solution. After concentration at 2500×g for 30s, cell suspensions were re-suspended in 100 ml of 0.5 % peptone salt solution for viable counts and dry weight determinations. Cell suspension without glycerol was used as the control. The water activity (a_w) was estimated after each addition of glycerol with Norrish equation:

$$a_w = (1\text{-}X)\, e^{-KX^2} \quad (3)$$

where a_w is water activity, X is the molar fraction glycerol, and K = 1.16 is the correlating constant for glycerol at 25°C.

Effect of Freezing on Viability of Tested Bacteria

Three ml aliquots of 18 h culture of each stock culture in 150 ml MRS broth were distributed into 15 ml Falcon® tubes, frozen and stored at -80 °C for 4h. The tubes were then defrosted in a water bath at 50°C for 10 min and cooled at room temperature (30°± 2°C) for 20 min.

Effect of Incubation Temperature on Viability of Tested Bacteria

Three ml aliquots of 18 h culture of each stock culture in 150 ml MRS broth were distributed into 15 ml Falcon® tubes, frozen and stored at -80 °C for 4h. The tubes were then defrosted in a water bath at 50°C for 10 min and cooled at room temperature (30°± 2°C) for 20 min.

Data Analyses

All inoculations and essays were carried out in duplicate and the average values were recorded. Viable counts and dry weight were determined after each stress treatment, and compared to those of the control (cell without treatment). Viable counts were expressed dry weight basis. The percentage survival of the strains before and after stress treatments was expressed as follows: Survival (%) = N_0/N_c ×100, where N_0 is the cfu/g DW before stress treatment and N_c is the cfu/g DW after stress treatment. Data were compared using Tukey's honestly significant difference and Student *t* test (Statistica 8.0, StatSoft Inc., 2007). The level of significance was set at P = 0.05.

RESULTS AND DISCUSSION

The selection of LAB as dried starter culture for cereal fermentation is a complex process, involving the evaluation of some desired metabolic traits and technological performances. Although the selection needs to take into account many parameters (acidification, production of antimicrobial compounds, viability after dehydration, etc.), this work proposes a first approach based upon kinetics growth parameters and the ability to overcome stressful conditions. Modeling of cell growth, acidification power as well as production of bacteriocins and exopolysaccharides could be used to predict LAB starter cultures bio-kinetics in food industry [5]. Primary growth models such as the maximal specific growth rate (μ_m) and the generation time (g) could be used to describe changes in microbial numbers (viability) or microbial responses with time [4]. The linear part of the growth logarithmic plot characterized the exponential growth (Fig. **1**). The slope of the straight line corresponded to μ_m (Equation 2, Table **1**). This graph clearly shows that exponential growth followed immediately the adaptation phase of the inoculated biomass to the physico-chemical conditions and medium, since growth became exponential after less than 3h. The generation time (g) is the time needed for doubling the initial bacterial population during the exponential growth. It is directly linked to the specific growth rate (Equation 1, Table **1**). The generation time values, which ranged from 0.61 to1.33 h, were compared to those (ranging from 0.5 to 2.3 h) of Beal *et al.* [6] reported for lactic acid bacteria on MRS medium. The maximal specific growth rate (1.14 h^{-1}) and g (0.61 h) were, significantly higher and lower for the strain *L. fermentum* (LF) compared with those obtained by the strains LP, M1 and M2, respectively.

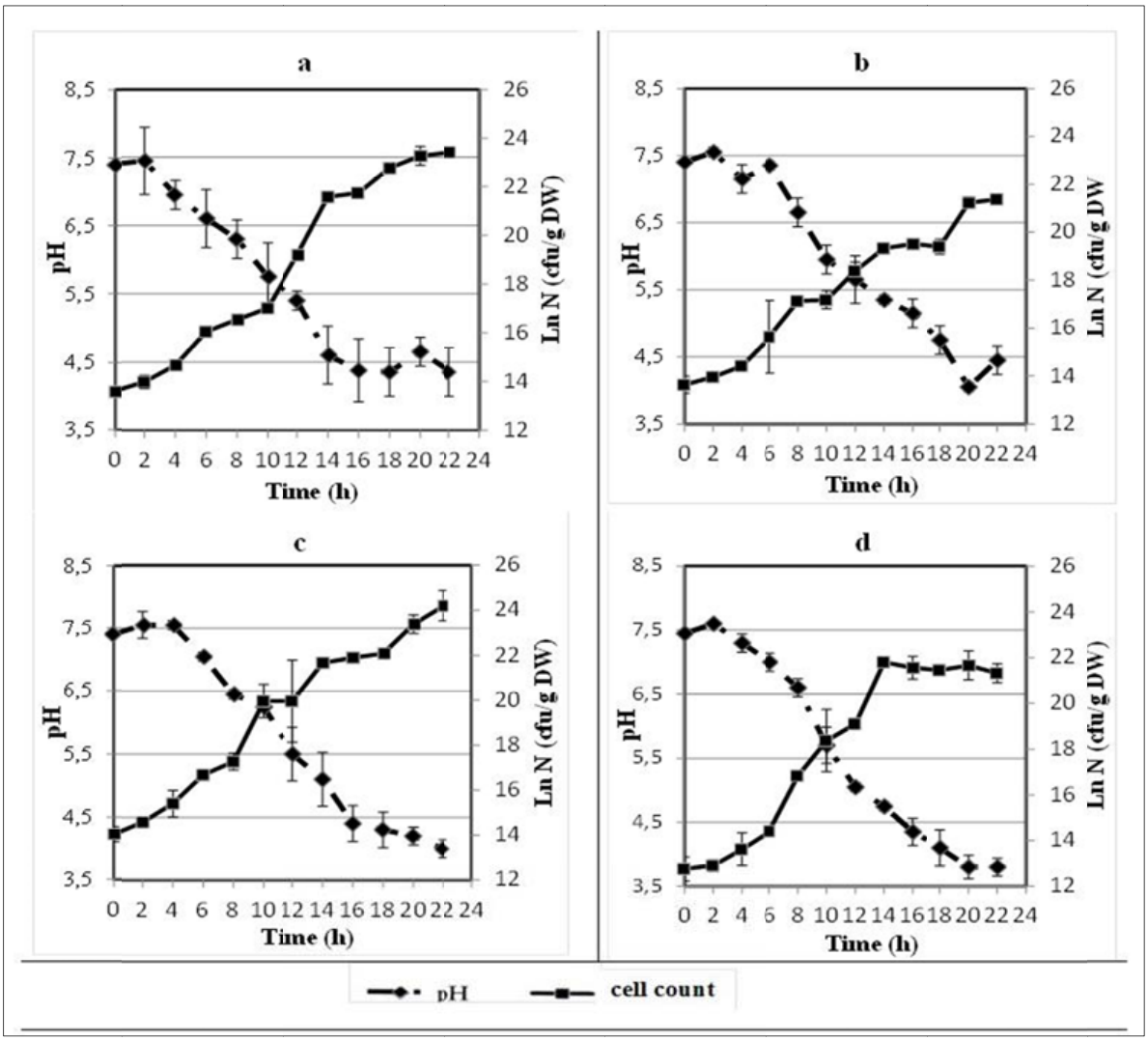

Fig (1). Viable cell counts (■) and pH values (♦) during fermentation with (**a**) *Lactobacillus fermentum* (LF), (**b**) *L. plantarum* (LP), (**c**) *Lactococcus* spp M1 (**d**) *Leuconostoc* spp M2. Values are presented as means ± Standard Deviation (SD) (n = 4).

Table 1. Growth and kinetic growth parameters after 18h culture of *Lactobacillus fermentum* (LF), *L. plantarum* (LP), *Lactococcus* spp M1, *Leuconostoc* spp M2 on MRS medium.

Strains	Growth parameters		Kinetic growth parameters	
	Viable counts (cfu/ml)	Dry weight (%)	μ_m (h^{-1})	g (h)
LF	$2.63 \times 10^8 \pm 6.01 \times 10^7$ [a]	3.39 ± 0.07 [a]	1.14 ± 0.03 [a]	0.61 ± 0.02 [a]
LP	$9.15 \times 10^6 \pm 2.62 \times 10^5$ [b]	3.39 ± 0.17 [a]	0.54 ± 0.08 [b]	1.33 ± 0.05 [b]
M1	$1.36 \times 10^8 \pm 8.84 \times 10^6$ [c]	3.49 ± 0.05 [a]	1.05 ± 0.05 [ab]	0.73 ± 0.08 [ab]
M2	$7.10 \times 10^7 \pm 8.70 \times 10^6$ [bc]	3.40 ± 0.13 [a]	0.57 ± 0.03 [b]	1.22 ± 0.06 [b]

cfu: Colony-forming units
μ_m: Maximal specific growth rate
g: Generation time
Values are presented as means ± SD (n = 4)
[a-c]: values not sharing the same superscript letter within a column are significantly different, $p = 0.05$ (Tukey HSD test, n = 4).

The ability of the 4 strains to overcome stressful conditions was assessed by viable counts after dehydration in a glycerol solution of increasing concentration (from 1 to 5 ml), heat and freezing stresses. An increase in glycerol concentration was associated with a decrease in water activity (a_w) from 0.8 to 0.3 (Equation 3, Table **2**). The impact of the decrease in a_w on the viability of the strains was studied after viable counts and was compared with that of the control (cells without dehydration). The change in a_w in the environment of the bacteria had a significant effect on their viability, and this varied from one strain to another. All the strains did not recover more than 50% of their population value, whereas the strain *L. fermentum* (L) showed a higher viability than the others strains. In an earlier study, most of lactic acid bacteria strains (12 out of 16) recovered more than 50% of their population value after dehydration in a glycerol solution with a final a_w of about 0.3 [3]. The results obtained and reported here are only approximate, since the final a_w of the water glycerol solution in our study (0.3) was higher than that obtained generally for freeze-dried lactic acid powders (0.1-0.2) [7]. Freezing had a significant effect on bacterial viability, and this varied from one strain to another, as shown in Table **3**. Significant loss in viability of *Lactobacillus delbrueckii* ssp. *bulgaricus* CFL1 after freeze thawing in MRS broth was reported [8]. This difference in cell viability between strains was linked to the intrinsic resistance of the strain, the initial concentration, growth conditions and possibly the elimination of a large part of the sensitive population during the freezing step [9]. The strains were sensitive to a variation in the incubation temperature as shown in Table **4**. This sensitivity to change in the incubation temperature was characterized by a loss of viability between 98% and 100% or about 100% at 54 ° C or 75 ° C, respectively. Significant effect of temperature on the viability of LAB used as starter cultures has already been reported [10, 11]. However, the effect of incubation temperature was less evident in our study. The elimination of the most sensitive part of cell population during the stress treatment could be suggested.

These results indicate that the kinetic growth parameters (μ_m, g) together with the viability after stress treatments could be used to predict the potential of LAB as dried starter culture. However, further attempts for the screening of LAB dried starter culture must be made.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflicts of interest.

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Table 2. Viable count and survival of strains after dehydration with a glycerol solution of increasing concentration.

Strains	Viable count (cfu/g DW)		Survival (%)
	Before treatment ($a_w = 1$)	After treatment ($a_w = 0.32$)*	
LF	$2.06 \times 10^{12} \pm 2.24 \times 10^{11}$	$4.43 \times 10^{11} \pm 4.48 \times 10^{10}$	20.95 ± 3.07^{a}
LP	$1.44 \times 10^{12} \pm 1.86 \times 10^{11}$	$2.49 \times 10^{11} \pm 6.81 \times 10^{10}$	17.42 ± 4.30^{b}
M1	$3.08 \times 10^{12} \pm 8.41 \times 10^{11}$	$3.73 \times 10^{11} \pm 6.88 \times 10^{10}$	12.57 ± 3.40^{c}
M2	$2.14 \times 10^{12} \pm 4.47 \times 10^{11}$	$3.41 \times 10^{11} \pm 7.45 \times 10^{10}$	16.26 ± 4.03^{b}

cfu: Colony-forming units
Values are presented as means ± SD (n = 4).
[a-c]: values not sharing the same superscript letter within a column are significantly different, $p = 0.05$ (Tukey HSD test, n = 4).
*Molar fraction of glycerol (5ml glycerol + 1 ml culture media) is 0.55

Table 3. Viable count and survival of strains after freezing stress.

Strains	Viable count (cfu/g DW)		Survival (%)
	Before treatment	After treatment	
LF	$3.11 \times 10^{11} \pm 3.56 \times 10^{10}$	$9.60 \times 10^{10} \pm 1.99 \times 10^{9}$	31.03 ± 6.02^{a}
LP	$1.03 \times 10^{12} \pm 1.06 \times 10^{11}$	$8.72 \times 10^{10} \pm 1.35 \times 10^{9}$	8.51 ± 1.30^{bc}
M1	$3.21 \times 10^{11} \pm 7.63 \times 10^{10}$	$3.73 \times 10^{9} \pm 9.60 \times 10^{8}$	1.20 ± 0.11^{b}
M2	$4.86 \times 10^{11} \pm 1.19 \times 10^{10}$	$7.73 \times 10^{10} \pm 1.57 \times 10^{9}$	16.38 ± 4.26^{c}

cfu: Colony-forming units
Values are presented as means ± SD (n = 4)
[a-c]: values not sharing the same superscript letter within a column are significantly different, $p = 0.05$ (Tukey HSD test, n = 4).

Table 4. Viable count and survival of strains after incubation at à 54°C or 75°C.

Strains	Viable count (cfu/g DW)			Survival (%)	
	Before treatment	After treatment			
		T = 45°C	T = 75°C	T = 45°C	T = 75°C
LF	$7.42 \times 10^{11} \pm 1.30 \times 10^{10}$	$3.08 \times 10^{9} \pm 1.45 \times 10^{8}$	$2.87 \times 10^{8} \pm 6.93 \times 10^{7}$	$0.42 \pm 0.17^{*\ a}$	$0.04 \pm 0.01^{**\ a}$
LP	$5.18 \times 10^{11} \pm 9.99 \times 10^{9}$	$4.74 \times 10^{9} \pm 1.56 \times 10^{8}$	$3.11 \times 10^{8} \pm 1.08 \times 10^{7}$	$0.92 \pm 0.15^{*\ b}$	$0.06 \pm 0.02^{**\ a}$
M1	$4.61 \times 10^{11} \pm 9.70 \times 10^{10}$	$7.72 \times 10^{9} \pm 1.63 \times 10^{8}$	$6.37 \times 10^{8} \pm 5.82 \times 10^{7}$	$1.71 \pm 0.30^{*\ c}$	$0.14 \pm 0.03^{**\ b}$
M2	$4.37 \times 10^{11} \pm 2.20 \times 10^{8}$	$3.05 \times 10^{9} \pm 2.89 \times 10^{6}$	$5.58 \times 10^{8} \pm 5.04 \times 10^{7}$	$0.70 \pm 0.05^{*\ ab}$	$0.13 \pm 0.09^{**\ b}$

T: Temperature (°C)
cfu: Colony-forming units
Values are presented as means ± SD (n = 4).
[a-c] : Values not sharing the same letter within a column are significantly different, $p = 0.05$ (Tukey HSD test, n = 4).
*Values sharing different superscript marks within a raw are significantly different $p = 0.05$ (Student *t* test, n = 4).

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