

Starter Culture Optimization for Cereal- and Millet-Based Fermented Beverages

Authors:

Vaidehi Mande^a, Sneha Karadbhajne^b

Affiliations:

¹ Department of Food Technology^{a,b},

L.I.T, Rashtrasant Tukadoji Maharaj Nagpur University, Nagpur , Maharashtra , India

Corresponding Author: **Vaidehi Mande^a**

Email id: vaidehimande@gmail.com

Abstract

This study compared natural starter cultures with a standardized mixed starter system for fermentation of white rice, red rice, finger millet, pearl millet, sorghum, and barley. Natural starters were prepared using processed cereal or millet flour, curd as microbial inoculum, and sugar as an additional fermentable substrate. Curd concentrations of 1, 5, and 10% combined with 1 or 2% sugar were evaluated at 37 °C. A mixed starter comprising equal proportions of presumptive culture components corresponding to *Lactobacillus acidophilus*, *Lactobacillus casei*, and *Bifidobacterium* was evaluated at 3, 4, and 5% inoculum levels. Natural starter performance was substrate dependent. The combination of 5% curd and 2% sugar was selected for all six substrates, requiring 12 h for white rice, red rice, and barley and 24 h for finger millet, pearl millet, and sorghum. Maximum specific growth rates ranged from 1.319 to 1.997 h⁻¹ among natural starters. The mixed starter reduced the selected fermentation period, with 5% inoculum reaching 2.19×10^{10} CFU/mL within 6 h, together with pH 4.35 ± 0.078 and titratable acidity of approximately 0.841% lactic acid. This treatment showed the highest maximum specific growth rate of 2.88 h⁻¹ and a calculated doubling time of 0.24 h. The results demonstrate matrix-dependent behaviour of natural starter systems and show that controlled mixed inoculation can provide rapid fermentation under the tested conditions. These optimized starter conditions provide a basis for further development and validation of cereal- and millet-based fermented beverages.

Keywords: cereal beverages; millet beverages; lactic acid bacteria; starter culture; fermentation kinetics; mixed culture; non-dairy beverages.

1. Introduction

Growing interest in plant-based and non-dairy foods has encouraged the development of fermented beverages from cereals, millets, legumes, nuts, and seeds. Cereals are particularly suitable fermentation substrates because they contain fermentable carbohydrates together with proteins, dietary fibre, minerals, vitamins, and phytochemicals. Fermentation can modify these components through microbial metabolism and can simultaneously influence acidity, flavour, texture, nutrient accessibility, and microbiological stability (Blandino et al. 2003; Petrova et al. 2022; Kumar et al. 2023).

Cereal fermentation has historically relied on spontaneous or naturally established microbial communities. Lactic acid bacteria (LAB) are among the predominant microorganisms associated with many fermented cereal foods and beverages and contribute to acidification through the conversion of fermentable carbohydrates into organic acids. LAB may also generate metabolites such as exopolysaccharides, aroma compounds, and antimicrobial compounds that affect product quality (Bintsis 2018). Nevertheless, LAB performance depends on the microbial strain, substrate composition, nutrient availability, fermentation temperature, inoculum size, and duration.

Rice, barley, sorghum, and millets provide markedly different fermentation environments. White rice is predominantly starch-rich and comparatively low in fibre, whereas red rice contains a greater proportion of bran-associated components. Finger millet and pearl millet provide substantial dietary fibre and minerals, sorghum contains phenolic

compounds and structurally complex carbohydrates, and barley is distinguished by its β -glucan-rich fibre fraction. Processing operations including soaking, germination, milling, cooking, and fermentation further modify nutrient accessibility and physical characteristics (Nanje Gowda et al. 2022). Consequently, starter cultures optimized in one cereal substrate cannot necessarily be assumed to perform identically in another.

Natural starter systems remain attractive because of their accessibility and compatibility with traditional fermentation practices. However, naturally derived microbial communities may show batch-to-batch variability in composition and fermentation behaviour. In contrast, deliberately inoculated starter cultures provide greater control over the initial microbial population and can potentially shorten fermentation time and improve process reproducibility (Tamang et al. 2016; Bintsis 2018).

The inoculum concentration represents an important process variable because it influences the initial microbial population, duration of the lag phase, acidification kinetics, substrate utilization, and time required to achieve the desired microbial density. Evaluation of endpoint viable counts alone therefore provides an incomplete description of fermentation performance. Growth-kinetic parameters such as specific growth rate (μ), maximum specific growth rate ($\mu_{\max}$), doubling time, and substrate-growth relationships provide additional information for comparing starter systems.

The present study was undertaken to develop and comparatively evaluate natural and standardized mixed starter cultures for cereal- and millet-based fermented beverages. White rice, red rice, finger millet, pearl millet, sorghum, and barley were evaluated as fermentation substrates. The objectives were to characterize the substrates, standardize curd-based natural starter cultures, optimize a mixed starter at different inoculum concentrations, compare microbial growth kinetics, and identify technologically suitable conditions for subsequent beverage development.

2. Materials and Methods

2.1 Raw materials

White rice, red rice, finger millet, pearl millet, sorghum, and barley were selected as substrates and procured from a local market in Nagpur, Maharashtra, India. Raw materials were selected based on grain uniformity, absence of visible fungal contamination and insect damage, and acceptable moisture condition.

2.2 Pre-processing of cereal and millet substrates

All grains were cleaned and graded to remove stones, dust, broken grains, immature kernels, and foreign material, and then washed with distilled water. For soaking standardization, 100 g portions were evaluated at grain-to-water ratios of 1:2, 1:3, 1:4, and 1:5 (w/v) at 25 ± 2 °C for 12 and 24 h. Rice was subsequently soaked for 12 h, whereas millet, sorghum, and barley samples were soaked for 24 h using a 1:5 (w/v) ratio. Finger millet, pearl millet, sorghum, and barley were germinated at approximately 25 ± 2 °C and 90–95% relative humidity. Finger millet and barley were selected after 24 h germination, while pearl millet and sorghum were germinated for 48 h. Germinated grains were dried at approximately 40 °C until moisture content was approximately 10–12%, followed by milling.

2.3 Preparation of natural starter cultures

Natural starter cultures were prepared independently from six grain substrates: white rice, red rice, finger millet, pearl millet, sorghum, and barley. The processed flour of each substrate was dispersed separately in distilled water at a flour-to-water ratio of 1:5 (w/v) to obtain a uniform slurry. Market curd was used as the microbial inoculum, and sugar was incorporated as an additional fermentable substrate. The formulations were mixed thoroughly under hygienic conditions and incubated at 37 °C. Each grain substrate was evaluated independently to account for differences in substrate composition and fermentation behavior.

2.4 Optimization and standardization of natural cultures

Optimization of the natural starter cultures was performed by evaluating different combinations of curd inoculum and sugar concentration. Curd was tested at 1, 5, and 10% (w/w), while sugar was incorporated at 1 and 2% (w/w),

resulting in six treatment combinations: T1, 1% curd + 1% sugar; T2, 1% curd + 2% sugar; T3, 5% curd + 1% sugar; T4, 5% curd + 2% sugar; T5, 10% curd + 1% sugar; and T6, 10% curd + 2% sugar. The formulations were incubated at 37 °C and evaluated at 12 and 24 h.

Starter-culture performance was assessed at the specified fermentation intervals based on pH, titratable acidity expressed as percentage lactic acid (% LA), viable presumptive lactic acid bacteria (LAB) count, gas formation, and evidence of undesirable microbial growth. The optimum treatment for each grain substrate was selected by considering adequate acidification, development of a high presumptive LAB population, fermentation duration, and absence of excessive acidification or undesirable fermentation characteristics. The selected natural starter cultures were refrigerated at 4 ± 1 °C and used within 24–48 h for subsequent beverage-fermentation experiments.

2.5 Preparation of mixed starter cultures

Commercial culture products containing the intended starter microorganisms were used as the microbial source. Appropriate serial dilutions of each culture were prepared aseptically and spread-plated on De Man, Rogosa and Sharpe (MRS) agar. The inoculated plates were incubated at 37 °C for approximately 24 h. Following incubation, morphologically distinct colonies were selected and transferred individually into sterile MRS broth for propagation. The cultures were grown at 37 °C for approximately 18–24 h and subsequently purified by repeated streaking on fresh MRS agar until morphologically uniform colonies were obtained.

The recovered isolates were preliminarily characterized based on colony morphology, Gram staining, and catalase reaction. Since molecular or validated species-specific identification was not performed, the recovered cultures were considered presumptive isolates corresponding to the microorganisms declared in the respective commercial culture products. The presumptive culture components corresponding to *Lactobacillus acidophilus* and *Lactobacillus casei*, together with a presumptive *Bifidobacterium* component were propagated separately in MRS broth at 37 °C for 18–24 h. Actively grown cultures were then combined aseptically in equal proportions (1:1:1, v/v/v) to prepare the mixed starter culture.

2.6 Optimization and standardization of mixed starter concentration

The mixed starter culture, prepared by combining the three separately propagated presumptive culture components corresponding to *Lactobacillus acidophilus* and *Lactobacillus casei*, together with a presumptive *Bifidobacterium* component in equal proportions (1:1:1, v/v/v), was evaluated at inoculum concentrations of 3, 4, and 5% (v/v). Each inoculum concentration was incubated at 37 °C for up to 12 h under controlled fermentation conditions. Samples were collected at 0, 3, 6, 9, and 12 h and analysed for presumptive LAB count, pH, and titratable acidity expressed as percentage lactic acid. The 0 h measurement represented the initial condition immediately after inoculation. Changes in presumptive LAB population, pH, and titratable acidity were monitored to compare microbial growth and acidification patterns among the different inoculum concentrations.

The optimum inoculum concentration and incubation period were selected by comparing the presumptive LAB growth and acidification profiles obtained during the optimization trials. Selection was based on rapid microbial proliferation, attainment of an adequate presumptive LAB population, appropriate acidification, and reduced fermentation duration. The term “presumptive LAB” was used because species-level identity of the recovered cultures was not confirmed by molecular or validated species-specific identification. Furthermore, the term “probiotic” was not applied to the recovered isolates because probiotic designation requires adequately characterized microorganisms with demonstrated health benefits when administered in sufficient amounts (Hill et al. 2014). The selected mixed starter composition, inoculum concentration, and incubation conditions were subsequently used for the preparation of cereal- and millet-based fermented beverages.

2.7 Growth-kinetic assessment

Microbial growth kinetics were evaluated using the viable presumptive lactic acid bacteria (LAB) counts obtained at defined intervals during fermentation. For the standardized mixed starter culture, viable counts were determined at 0, 3, 6, 9, and 12 h. The viable-count data, expressed as CFU/mL, were used to determine the specific growth rate (μ), maximum specific growth rate ($\mu_{\max}$), and doubling time (t_d). These parameters were selected to compare the rate of microbial proliferation among the different starter systems and inoculum concentrations. The specific growth rate (μ) for each consecutive fermentation interval was calculated from the change in viable cell population according to the following equation:

$$\mu = [\ln(N_2) - \ln(N_1)] / (t_2 - t_1)$$

where N_1 and N_2 are the viable presumptive LAB populations (CFU/mL) measured at the beginning and end of a given fermentation interval, respectively, and t_1 and t_2 are the corresponding fermentation times (h). The specific growth rate was expressed as h^{-1} . The maximum specific growth rate ($\mu_{\max}$) was defined as the highest interval-specific μ value obtained during the monitored fermentation period. The fermentation interval corresponding to $\mu_{\max}$ was recorded to identify the period of most rapid microbial proliferation. The doubling time (t_d), representing the theoretical time required for the microbial population to double during the period of maximum growth, was calculated from $\mu_{\max}$ according to the equation:

$$t_d = \ln(2) / \mu_{\max}$$

where t_d is expressed in hours and $\mu_{\max}$ in h^{-1} . Kinetic calculations were performed separately for each natural cereal- and millet-based starter culture and for the 3, 4, and 5% (v/v) standardized mixed-starter treatments to allow comparison of their growth behaviour under the evaluated fermentation conditions.

A Monod-type kinetic analysis was considered during the initial evaluation; however, reliable estimation of the Monod half-saturation constant (K_s) requires quantitative measurements of the concentration of the growth-limiting substrate during fermentation. Because time-resolved residual fermentable-substrate concentrations were not determined in the present study, the available data were considered insufficient for a defensible Monod-model fit. Consequently, apparent K_s values were excluded from the final kinetic analysis. Growth-kinetic interpretation was therefore restricted to parameters directly supported by the experimental viable-count data, namely μ , $\mu_{\max}$, doubling time, and the fermentation interval corresponding to $\mu_{\max}$.

2.8 Physicochemical and microbiological analyses

pH was measured using a calibrated digital pH meter. Titratable acidity was determined by titration with standardized 0.1 N NaOH using phenolphthalein as the endpoint indicator and expressed as percentage lactic acid. Viable presumptive LAB populations were enumerated on MRS agar and expressed as CFU/mL. Yeast and mould and coliform observations were included as microbiological quality indicators where applicable.

2.9 Statistical treatment

Analytical measurements were performed in triplicate and expressed as mean $\pm$ standard deviation where replicate values were available. Interpretation focused on experimentally supported differences and kinetic trends. Inferential statistical conclusions were not introduced where complete independent batch-level data were unavailable.

3. Results and Discussion

3.1 Characteristics of curd used as the natural inoculum

The curd selected as the natural microbial inoculum had a pH of 4.66 ± 0.029 and titratable acidity of $0.740 \pm 0.080\%$ lactic acid. Total soluble solids were 10.9 ± 0.009 °Brix, while viscosity was 1899 ± 0.106 cP. The proximate composition comprised $87.9 \pm 0.098\%$ moisture, $4.26 \pm 0.035\%$ fat, $5.01 \pm 0.065\%$ protein, and $0.88 \pm 0.009\%$ ash. Microbiological evaluation showed a standard plate count (SPC) of 3.1×10^3 CFU/mL and a presumptive LAB population of 22×10^5 CFU/mL, whereas yeast, mould, and coliforms were not detected. The combination of an acidic

environment and substantial presumptive LAB population supported the technological use of the curd as a natural inoculum. LAB play a central role in food fermentation primarily through carbohydrate metabolism and organic-acid production, while additional metabolites can contribute to flavour, texture, and microbial interactions (Bintsis 2018). This interpretation is consistent with the established technological role of LAB as acidifying starter organisms in fermented foods (Leroy and De Vuyst 2004; Bintsis 2018; Tamang et al. 2016).

Table 1. Characteristics of curd used as natural inoculum

Parameter	Result
pH	4.66 ± 0.029
Titratable acidity (% LA)	0.740 ± 0.080
TSS (°Brix)	10.9 ± 0.009
Viscosity (cP)	1899 ± 0.106
Moisture (%)	87.9 ± 0.098
Fat (%)	4.26 ± 0.035
Protein (%)	5.01 ± 0.065
Ash (%)	0.88 ± 0.009
SPC (CFU/mL)	3.1 × 10 ³
Presumptive LAB (CFU/mL)	22 × 10 ⁵
Yeast and mould	Not detected
Coliforms	Not detected

3.2 Composition and functional characteristics of cereal and millet substrates

Substantial compositional variation was observed among the six grain matrices. Moisture ranged from 9.76 ± 0.11% in pearl millet to 11.70 ± 0.14% in red rice. Protein ranged from 7.70 ± 0.08% in white rice to 12.01 ± 0.12% in barley. Pearl millet contained the greatest fat concentration (2.82 ± 0.05%), while white rice contained 0.50 ± 0.12%. Dietary fibre showed pronounced differences, ranging from 1.82 ± 0.04% in white rice to 10.76 ± 0.12% in pearl millet. Conversely, carbohydrate concentration was highest in white rice (76.51 ± 0.20%) and red rice (71.69 ± 0.18%). Such compositional heterogeneity is technologically important because LAB growth in cereal systems depends on accessibility of fermentable carbohydrates, nitrogen sources, minerals, and other growth factors. Processing operations such as soaking, germination, milling, and fermentation can further modify the accessibility of these components (Nanje Gowda et al. 2022). Comparable substrate-dependent effects on cereal fermentation have been described in the literature (Blandino et al. 2003; Petrova et al. 2022; Kumar et al. 2023).

Table 2. Selected compositional characteristics of cereal and millet substrates

Parameter	White rice	Red rice	Finger millet	Pearl millet	Sorghum	Barley
Moisture (%)	11.08 ± 0.12	11.70 ± 0.14	10.98 ± 0.10	9.76 ± 0.11	9.87 ± 0.09	10.35 ± 0.10
Fat (%)	0.50 ± 0.12	1.32 ± 0.03	1.86 ± 0.04	2.82 ± 0.05	2.23 ± 0.04	1.30 ± 0.03
Protein (%)	7.70 ± 0.08	7.88 ± 0.07	9.30 ± 0.09	9.36 ± 0.10	10.31 ± 0.11	12.01 ± 0.12
Ash (%)	0.52 ± 0.01	1.34 ± 0.02	1.19 ± 0.02	1.69 ± 0.03	1.21 ± 0.02	1.81 ± 0.03
Dietary fibre (%)	1.82 ± 0.04	3.03 ± 0.05	10.30 ± 0.11	10.76 ± 0.12	9.56 ± 0.10	10.20 ± 0.11

Carbohydrate (%)	76.51 ± 0.20	71.69 ± 0.18	66.37 ± 0.16	54.87 ± 0.15	56.98 ± 0.17	54.13 ± 0.14
Amylose (%)	21.84 ± 0.30	25.88 ± 0.34	17.89 ± 0.27	20.30 ± 0.29	25.89 ± 0.33	29.77 ± 0.36
Amylopectin (%)	78.16 ± 0.82	74.12 ± 0.74	82.11 ± 0.83	79.70 ± 0.79	74.11 ± 0.72	69.23 ± 0.70
Cooking time (min)	33.34 ± 0.55	51.67 ± 0.62	24.70 ± 0.50	27.81 ± 0.53	44.22 ± 0.60	54.32 ± 0.64

3.3 Selected conditions and characteristics of natural starter cultures

Optimization demonstrated a clear influence of curd and sugar concentration on the fermentation characteristics of the cereal- and millet-based natural starter cultures. In general, increasing the curd concentration enhanced microbial proliferation and acidification. Treatments containing 1% curd showed comparatively limited fermentation, whereas the 10% curd treatments generally produced substantially greater acidification. The intermediate 5% curd treatments provided a more balanced combination of microbial growth and acid development. Based on the combined evaluation of pH, titratable acidity and viable microbial population, T4 (5% curd + 2% sugar) was selected as the optimum treatment for all six substrates. However, the fermentation time required to reach the selected condition was substrate dependent.

White rice, red rice and barley reached their selected conditions after 12 h, whereas finger millet, pearl millet and sorghum required 24 h. The selected cultures had pH values of 4.37–4.47 and titratable acidity values of approximately 0.69–0.86% LA. The viable populations ranged from 5.9×10^8 CFU/mL for sorghum to 8.6×10^9 CFU/mL for pearl millet. These results demonstrate that identical curd and sugar concentrations did not produce identical fermentation behaviour across the grain matrices, supporting substrate-specific selection of fermentation duration. Matrix-dependent fermentation behaviour is consistent with reports that cereal composition and nutrient availability influence LAB activity and fermentation performance (Blandino et al. 2003; Tamang et al. 2016; Petrova et al. 2022).

Table 3. Optimized natural starter culture conditions for selected cereal and millet substrates

Substrate	Curd (%)	Sugar (%)	Time (h)	pH	TA (% LA)	Viable count (CFU/mL)
White rice	5	2	12	4.40	0.72	3.8×10^9
Red rice	5	2	12	4.42	0.69	2.9×10^9
Finger millet	5	2	24	4.40	0.86	2.0×10^9
Pearl millet	5	2	24	4.38	0.86	8.6×10^9
Sorghum	5	2	24	4.37	0.80	5.9×10^8
Barley	5	2	12	4.47	0.82	2.9×10^9

3.4 Selection of the Standardized Mixed Starter Culture

The fermentation characteristics of the mixed starter culture were influenced by both inoculum concentration and incubation time (Table 4). Across all three inoculum levels, fermentation was characterized by a progressive decrease in pH, an increase in titratable acidity, and substantial growth of presumptive LAB. However, the rate at which these changes occurred differed among the 3, 4, and 5% inoculum levels, indicating that the initial starter concentration affected the time required to achieve the desired fermentation characteristics.

At 3% inoculum, the initial pH was 6.50 ± 0.021 , with a titratable acidity of $0.311 \pm 0.024\%$ LA and a presumptive LAB count of 1.1×10^3 CFU/mL. During fermentation, the pH progressively decreased to 5.40 ± 0.080 at 3 h, 4.92 ± 0.011 at 6 h, and 4.57 ± 0.118 at 9 h, finally reaching 4.44 ± 0.023 at 12 h. Correspondingly, titratable acidity increased from $0.311 \pm 0.024\%$ to $0.720 \pm 0.013\%$ LA. Presumptive LAB counts increased substantially from 1.1×10^3 CFU/mL initially to 3.9×10^5 , 4.0×10^7 , 2.22×10^9 , and 5.11×10^9 CFU/mL at 3, 6, 9, and 12 h, respectively. Thus, the 3% inoculum required approximately 12 h to achieve the selected combination of microbial population and acidification. Increasing the inoculum level to 4% accelerated fermentation. The initial presumptive LAB population was 2.5×10^4 CFU/mL at pH 6.10 ± 0.022 and titratable acidity of $0.341 \pm 0.230\%$ LA. The population increased to 5.4×10^5 CFU/mL after 3 h and 1.1×10^8 CFU/mL after 6 h. At 9 h, the presumptive LAB count reached 1.39×10^9 CFU/mL, accompanied by a pH of 4.48 ± 0.051 and titratable acidity of $0.750 \pm 0.040\%$ LA. Although extending fermentation to 12 h further increased the population to 3.2×10^{10} CFU/mL, pH decreased to 4.30 ± 0.075 and titratable acidity increased to $0.860 \pm 0.076\%$ LA. Therefore, 9 h was considered the selected fermentation point for the 4% inoculum because the culture had already achieved substantial microbial growth with a comparatively moderate degree of acidification.

The most rapid fermentation response was observed with the 5% inoculum. At 0 h, the culture showed a pH of 5.70 ± 0.013 , titratable acidity of $0.401 \pm 0.083\%$ LA, and a presumptive LAB count of 5.0×10^4 CFU/mL. After only 3 h, the population increased to 3.9×10^6 CFU/mL, while pH decreased to 4.65 ± 0.034 . A pronounced increase in microbial population occurred between 3 and 6 h, with the presumptive LAB count reaching 2.19×10^{10} CFU/mL at 6 h. At this point, the pH was 4.35 ± 0.078 and titratable acidity was $0.841 \pm 0.189\%$ LA. These values represented a favourable combination of rapid microbial proliferation and acidification within a relatively short fermentation period. Further incubation of the 5% culture to 9 h resulted in only a marginal increase in presumptive LAB count to 2.2×10^{10} CFU/mL, whereas pH declined further to 4.19 ± 0.092 and titratable acidity increased to $0.899 \pm 0.677\%$ LA. By 12 h, the presumptive LAB population had decreased to 7.9×10^9 CFU/mL, while the pH reached 4.07 ± 0.082 and titratable acidity increased to $0.935 \pm 0.099\%$ LA. This decline in viable population, together with continued acidification, suggests that prolonged fermentation conditions became less favourable for cell survival. Similar reductions in viability during prolonged acidic fermentation have been associated with acid stress and other matrix-dependent environmental constraints (Tripathi and Giri 2014; Terpou et al. 2019).

Overall, increasing the mixed starter concentration reduced the time required to obtain a high presumptive LAB population and the desired acidification profile. Although the 4% treatment produced a higher count of 3.2×10^{10} CFU/mL after 12 h, selection was not based solely on the maximum count observed at the end of fermentation. The 5% inoculum at 6 h was selected as the standardized condition, as it achieved a high presumptive LAB population of 2.19×10^{10} CFU/mL together with a pH of 4.35 ± 0.078 and titratable acidity of $0.841 \pm 0.189\%$ LA in substantially less time. This condition was therefore considered the most appropriate combination of microbial proliferation, controlled acidification, and reduced fermentation duration for subsequent cereal- and millet-based beverage experiments. Inoculum level is an important process variable because a higher initial cell population can alter fermentation kinetics and the time required to achieve acidification (Leroy and De Vuyst 2004; Bintsis 2018).

Table 4. Changes in fermentation characteristics of the mixed starter culture at different inoculum concentrations during 12 h incubation

Inoculum level (%) v/v)	Incubation time (h)	pH	Titratable acidity (%) LA)	Presumptive LAB count (CFU/mL)
3	0	6.50 ± 0.021	0.311 ± 0.024	1.1×10^3
	3	5.40 ± 0.080	0.487 ± 0.021	3.9×10^5
	6	4.92 ± 0.011	0.610 ± 0.039	4.0×10^7
	9	4.57 ± 0.118	0.684 ± 0.017	2.22×10^9

		0.118		
	12	4.44 ± 0.023	0.720 ± 0.013	5.11 × 10 ⁹
4	0	6.10 ± 0.022	0.341 ± 0.230	2.5 × 10 ⁴
	3	5.00 ± 0.029	0.499 ± 0.018	5.4 × 10 ⁵
	6	4.78 ± 0.047	0.690 ± 0.341	1.1 × 10 ⁸
	9	4.48 ± 0.051	0.750 ± 0.040	1.39 × 10 ⁹
	12	4.30 ± 0.075	0.860 ± 0.076	3.2 × 10 ¹⁰
5	0	5.70 ± 0.013	0.401 ± 0.083	5.0 × 10 ⁴
	3	4.65 ± 0.034	0.522 ± 0.074	3.9 × 10 ⁶
	6	4.35 ± 0.078	0.841 ± 0.189	2.19 × 10 ¹⁰
	9	4.19 ± 0.092	0.899 ± 0.677	2.2 × 10 ¹⁰
	12	4.07 ± 0.082	0.935 ± 0.099	7.9 × 10 ⁹

3.5 Growth kinetics of natural starter cultures

Growth-kinetic evaluation revealed marked substrate-dependent differences among the natural starter cultures (Table 5). The maximum specific growth rate ($\mu_{\max}$) varied from 1.319 to 1.997 h⁻¹, while the calculated doubling time ranged from 0.35 to 0.53 h. Such variation indicates that the cereal or millet matrix influenced the rate and timing of microbial proliferation. This is consistent with the broader understanding that cereal fermentations provide compositionally diverse environments in which microbial activity depends on the availability of fermentable carbohydrates, nutrients, and other matrix-specific factors (Tamang et al., 2016; Petrova et al., 2022).

Among the substrates, sorghum exhibited the highest $\mu_{\max}$ of 1.997 h⁻¹ during the 6–9 h interval and consequently the shortest doubling time of 0.35 h. Its presumptive LAB population increased from 3.0 × 10⁴ CFU/mL initially to 4.4 × 10¹⁰ CFU/mL after 12 h. Red rice exhibited the second-highest $\mu_{\max}$ (1.858 h⁻¹), with its maximum growth rate occurring earlier, during 0–3 h. The population increased from 2.2 × 10⁵ to 9.1 × 10¹⁰ CFU/mL, representing the highest 12-h count among the six substrates. White rice showed a $\mu_{\max}$ of 1.713 h⁻¹ during 3–6 h and a doubling time of 0.40 h, with the population increasing from 5.8 × 10⁵ to 1.2 × 10¹⁰ CFU/mL by 12 h.

Finger millet and barley exhibited intermediate maximum specific growth rates of 1.607 and 1.628 h⁻¹, respectively, with corresponding doubling times of 0.43 h. However, their periods of maximum growth differed: $\mu_{\max}$ occurred during 6–9 h for finger millet and 9–12 h for barley. Pearl millet showed the lowest $\mu_{\max}$ (1.319 h⁻¹) and the longest doubling time (0.53 h), although its presumptive LAB population still increased from 4.4 × 10⁴ to 2.6 × 10¹⁰ CFU/mL by 12 h. The different timings of $\mu_{\max}$ —from 0–3 h in red rice and pearl millet to 9–12 h in barley—indicate that the cultures did not follow identical growth trajectories despite being fermented under comparable conditions.

These differences are biologically plausible because cereal and millet matrices differ in their carbohydrate composition, nutrient accessibility, buffering capacity, and other physicochemical characteristics that can affect LAB growth and acidification. Reviews of cereal fermentation similarly emphasize that LAB performance is strongly dependent on the characteristics of the fermentation substrate and the organisms involved (Tamang et al., 2016; Petrova et al., 2022). Studies of cereal-based fermentation have also demonstrated that microbial growth patterns can

differ substantially across cereal matrices and LAB cultures. Such matrix effects are widely recognized in cereal fermentations (Blandino et al. 2003; Tamang et al. 2016; Petrova et al. 2022).

Importantly, the results demonstrate that the microbial count measured at a single endpoint does not fully describe fermentation performance. For example, red rice produced the highest population at 12 h, whereas sorghum exhibited the highest calculated $\mu_{\max}$ and shortest doubling time. Evaluation of $\mu_{\max}$, doubling time, the interval at which maximum growth occurred, and the apparent K_s therefore provides additional information about culture behaviour beyond final viable counts. Growth-kinetic modelling of LAB likewise uses parameters such as maximum specific growth rate and Monod-type constants to characterize microbial proliferation and its relationship with fermentation conditions (Burgos-Rubio et al., 2000; Amrane and Prigent, 1998).

Table 5. Growth-kinetic parameters of natural starter cultures derived from selected cereal and millet substrates

Substrate	Initial count	Count at 12 h	$\mu_{\max}$ (h^{-1})	Apparent K_s	Doubling time (h)	$\mu_{\max}$ interval
White rice	5.8×10^5	1.2×10^{10}	1.713	1.96	0.40	3–6 h
Red rice	2.2×10^5	9.1×10^{10}	1.858	1.94	0.37	0–3 h
Finger millet	2.9×10^5	5.1×10^{10}	1.607	1.90	0.43	6–9 h
Pearl millet	4.4×10^4	2.6×10^{10}	1.319	0.53	0.53	0–3 h
Sorghum	3.0×10^4	4.4×10^{10}	1.997	2.07	0.35	6–9 h
Barley	9.0×10^4	3.7×10^{10}	1.628	1.85	0.43	9–12 h

3.6 Growth kinetics of the standardized mixed starter culture

The growth-kinetic characteristics of the standardized mixed starter showed a clear inoculum-dependent response (Table 6). Increasing the inoculum concentration influenced both the rate of presumptive LAB proliferation and the time required to achieve a high viable population. At 3% inoculum, the population increased from 1.1×10^3 CFU/mL at 0 h to 5.11×10^9 CFU/mL after 12 h. The calculated $\mu_{\max}$ was 1.960 h^{-1} , with an apparent K_s of 0.75 and a doubling time of approximately 0.35 h. The data show progressive growth throughout the 12-h fermentation period, indicating that the lower inoculum required a comparatively longer incubation period to attain a high microbial population.

At 4% inoculum, the initial population was higher at 2.5×10^4 CFU/mL and increased to 3.2×10^{10} CFU/mL after 12 h. The $\mu_{\max}$ was 1.772 h^{-1} , with an apparent K_s of 1.26 and a calculated doubling time of 0.39 h. Although this treatment produced the highest 12-h population among the three inoculum levels, the culture required the complete 12-h incubation period to attain this count. At 9 h, the population was 1.39×10^9 CFU/mL, demonstrating that substantial additional growth occurred between 9 and 12 h.

The 5% inoculum exhibited the most rapid kinetic response. The presumptive LAB population increased from 5.0×10^4 CFU/mL at 0 h to 3.9×10^6 CFU/mL at 3 h and reached 2.19×10^{10} CFU/mL after only 6 h. The calculated $\mu_{\max}$ of 2.880 h^{-1} was the highest among the three inoculum concentrations, while the corresponding doubling time of 0.24 h was the shortest. The apparent K_s was 1.98. The population increased only marginally thereafter, reaching 2.2×10^{10} CFU/mL at 9 h, before declining to 7.9×10^9 CFU/mL at 12 h. The data concurrently show continued acidification after 6 h, with pH decreasing from 4.35 ± 0.078 at 6 h to 4.07 ± 0.082 at 12 h and titratable acidity increasing from 0.841 ± 0.189 to $0.935 \pm 0.099\%$ LA.

Overall, the kinetic parameters demonstrate that the highest endpoint population did not necessarily correspond to the most efficient fermentation condition. Although the 4% inoculum reached 3.2×10^{10} CFU/mL after 12 h, the 5% inoculum attained 2.19×10^{10} CFU/mL within only 6 h and exhibited the highest $\mu_{\max}$ and shortest doubling time. Therefore, the kinetic evaluation supports the selection of 5% inoculum with 6 h incubation as the standardized mixed starter condition, based on rapid microbial proliferation, high presumptive LAB population, appropriate acidification, and reduced fermentation duration.

Table 6. Growth-kinetic parameters of the standardized mixed starter culture at different inoculum concentrations

Inoculum	Initial count	Relevant count	Time	$\mu_{\max}$ (h ⁻¹)	Apparent Ks	Doubling time
3%	1.1×10^3	5.11×10^9	12 h	1.960	0.75	0.35 h
4%	2.5×10^4	3.2×10^{10}	12 h	1.772	1.26	0.39 h
5%	5.0×10^4	2.19×10^{10}	6 h	2.880	1.98	0.24 h

3.7 Comparative performance of natural and standardized starter systems

Comparative evaluation demonstrated that both the natural and standardized mixed starter systems supported substantial microbial proliferation, but their fermentation kinetics and processing requirements differed considerably. The natural starter cultures showed pronounced substrate-dependent behaviour. Under the selected natural starter condition of 5% curd and 2% sugar, white rice, red rice, and barley required 12 h of fermentation, whereas finger millet, pearl millet, and sorghum required 24 h to attain the selected fermentation characteristics. At these endpoints, pH ranged from 4.37 to 4.47 and titratable acidity from 0.69 to 0.86% LA. Presumptive LAB populations ranged from 5.9×10^8 CFU/mL in sorghum to 8.6×10^9 CFU/mL in pearl millet. These differences indicate that the fermentation performance of the natural starter was strongly influenced by the cereal or millet matrix.

Growth-kinetic analysis further demonstrated differences among the natural starter cultures. The calculated $\mu_{\max}$ values ranged from 1.319 h⁻¹ for pearl millet to 1.997 h⁻¹ for sorghum, while doubling times ranged from 0.35 to 0.53 h. Sorghum exhibited the highest $\mu_{\max}$ (1.997 h⁻¹) and shortest doubling time (0.35 h), whereas red rice attained the highest 12-h population of 9.1×10^{10} CFU/mL with a $\mu_{\max}$ of 1.858 h⁻¹. The interval at which $\mu_{\max}$ occurred also differed among substrates, ranging from 0–3 h for red rice and pearl millet to 9–12 h for barley. These observations demonstrate that the natural starter cultures did not exhibit a uniform growth pattern across the six grain matrices.

In comparison, the standardized mixed starter showed a clear inoculum-dependent acceleration of fermentation. At 3% inoculum, the presumptive LAB population increased from 1.1×10^3 to 5.11×10^9 CFU/mL over 12 h, with a $\mu_{\max}$ of 1.960 h⁻¹ and a doubling time of 0.35 h. At 4%, the population increased from 2.5×10^4 to 3.2×10^{10} CFU/mL after 12 h, with a $\mu_{\max}$ of 1.772 h⁻¹ and a doubling time of 0.39 h. The 5% inoculum produced the most rapid kinetic response, reaching 2.19×10^{10} CFU/mL after only 6 h from an initial population of 5.0×10^4 CFU/mL. The corresponding $\mu_{\max}$ was 2.880 h⁻¹ and the calculated doubling time was 0.24 h. At the selected 6-h endpoint, the pH was 4.35 ± 0.078 and titratable acidity was $0.841 \pm 0.189\%$ LA.

Thus, the principal technological distinction between the two starter systems was the time required to attain a high microbial population under the selected fermentation conditions. The natural starters required 12–24 h, depending on the substrate, whereas the standardized 5% mixed starter attained 2.19×10^{10} CFU/mL within 6 h. Its $\mu_{\max}$ of 2.880 h⁻¹ was also higher than the $\mu_{\max}$ values calculated for the natural starter cultures (1.319–1.997 h⁻¹), while its doubling time of 0.24 h was shorter than the corresponding range of 0.35–0.53 h for the natural cultures. These kinetic characteristics indicate faster microbial proliferation under the selected standardized mixed-starter condition. Controlled starter inoculation is generally used to improve fermentation reproducibility and process control relative to less-defined natural microbial systems (Leroy and De Vuyst 2004; Bintsis 2018; Tamang et al. 2016).

3.8 Application in cereal- and millet-based fermented beverage development

The optimized natural and standardized mixed starter systems provide a practical fermentation framework for the development of cereal- and millet-based fermented beverages. All six substrates supported substantial presumptive LAB proliferation; however, differences in $\mu_{\max}$, doubling time, acidification pattern, and selected fermentation duration demonstrated that microbial performance was matrix dependent. The natural starter containing 5% curd and 2% sugar required 12 h for white rice, red rice, and barley and 24 h for finger millet, pearl millet, and sorghum. These differences indicate that fermentation endpoints should be determined according to the individual substrate using microbial population, pH, titratable acidity, and fermentation time rather than applying a single fixed fermentation duration to all cereal and millet matrices.

The standardized mixed starter provided a more rapid fermentation strategy. At 5% inoculum, the presumptive LAB population reached 2.19×10^{10} CFU/mL within 6 h, with a pH of 4.35 ± 0.078 and titratable acidity of $0.841 \pm 0.189\%$ LA. Its $\mu_{\max}$ of 2.880 h^{-1} and doubling time of 0.24 h further demonstrated rapid microbial proliferation under the selected conditions. Compared with the 12–24 h required for the selected natural starter conditions, this shorter fermentation period could reduce production cycle time and facilitate more precise endpoint control. Standardization of inoculum concentration, incubation temperature, and fermentation duration may also improve batch-to-batch process consistency. Importantly, extending fermentation beyond the selected endpoint resulted in continued acidification without a proportional improvement in microbial population, emphasizing the need to control fermentation time to avoid over-acidification. The use of defined starter cultures can facilitate controlled acidification and more reproducible processing in fermented food systems (Leroy and De Vuyst 2004; Bintsis 2018). Process standardization is a central advantage of controlled starter-culture fermentation (Leroy and De Vuyst 2004; Bintsis 2018). Maintaining viability during fermentation and subsequent storage is known to depend on acidity, matrix composition, processing conditions, and strain characteristics (Tripathi and Giri 2014; Terpou et al. 2019).

These optimized starter conditions also provide a defined basis for subsequent beverage formulation and quality evaluation. Controlled fermentation can facilitate systematic assessment of formulation variables such as cereal-to-plant-milk ratio, stabilizer concentration, homogenization conditions, and other ingredients while minimizing variation arising from the starter system. Because fermentation-induced changes in pH and acidity can influence flavour, texture, viscosity, microbial quality, and product stability, microbial population should be considered together with physicochemical and sensory characteristics when selecting the final formulation. The optimized cultures therefore provide a process framework for subsequent evaluation of physicochemical properties, sensory acceptability, presumptive LAB retention, microbiological quality, and refrigerated storage stability of cereal- and millet-based fermented beverages. Fermentation-driven changes in physicochemical, sensory, and functional properties are also reported for cereal- and plant-based beverage systems (Kumar et al. 2023; Mefleh et al. 2024).

4. Conclusion

Natural and standardized mixed starter systems were successfully evaluated for fermentation of six cereal and millet matrices. The natural starter containing 5% curd and 2% sugar showed substrate-dependent fermentation, requiring 12 h for white rice, red rice, and barley and 24 h for finger millet, pearl millet, and sorghum. Growth-kinetic analysis further demonstrated differences among the grain matrices. In comparison, the 5% mixed starter achieved a presumptive LAB population of 2.19×10^{10} CFU/mL within 6 h, with pH 4.35 ± 0.078 , titratable acidity of approximately 0.841% lactic acid, $\mu_{\max}$ of 2.88 h^{-1} , and a calculated doubling time of 0.24 h. These findings demonstrate the importance of substrate-specific fermentation conditions and show that controlled mixed inoculation can shorten fermentation under the tested conditions. Further independent-batch validation, molecular confirmation of starter organisms, strain-specific enumeration, substrate-consumption analysis, sensory evaluation, and storage-stability studies are required before strain-specific probiotic or commercial shelf-life claims can be established.

5. References

1. Bintsis T (2018) Lactic acid bacteria as starter cultures: an update in their metabolism and genetics. *AIMS Microbiology* 4(4):665–684. <https://doi.org/10.3934/microbiol.2018.4.665>
2. Blandino A, Al-Aseeri ME, Pandiella SS, Cantero D, Webb C (2003) Cereal-based fermented foods and beverages. *Food Research International* 36(6):527–543. [https://doi.org/10.1016/S0963-9969\(03\)00009-7](https://doi.org/10.1016/S0963-9969(03)00009-7)
3. Hill C, Guarner F, Reid G, Gibson GR, Merenstein DJ, Pot B, Morelli L, Canani RB, Flint HJ, Salminen S, Calder PC, Sanders ME (2014) Expert consensus document: the International Scientific Association for Probiotics and Prebiotics consensus statement on the scope and appropriate use of the term probiotic. *Nature Reviews Gastroenterology & Hepatology* 11(8):506–514. <https://doi.org/10.1038/nrgastro.2014.66>
4. Patra M, Bashir O, Amin T, Wani AW, Shams R, Chaudhary KS, Mirza AA, Manzoor S (2023) A comprehensive review on functional beverages from cereal grains—characterization of nutraceutical potential, processing technologies and product types. *Heliyon* 9(6):e16804. <https://doi.org/10.1016/j.heliyon.2023.e16804>
5. Leroy F, De Vuyst L (2004) Functional lactic acid bacteria starter cultures for the food fermentation industry. *Trends in Food Science & Technology* 15(2):67–78. <https://doi.org/10.1016/j.tifs.2003.09.004>
6. Mefleh M, Omri G, Limongelli R, Minervini F, Santamaria M, Faccia M (2024) Enhancing nutritional and sensory properties of plant-based beverages: a study on chickpea and Kamut® flours fermentation using *Lactococcus lactis*. *Frontiers in Nutrition* 11:1269154. <https://doi.org/10.3389/fnut.2024.1269154>
7. Nanje Gowda NA, Siliveru K, Prasad PVV, Bhatt Y, Netravati BP, Gurikar C (2022) Modern processing of Indian millets: a perspective on changes in nutritional properties. *Foods* 11(4):499. <https://doi.org/10.3390/foods11040499>
8. Petrova P, Arsov A, Petrov K (2022) Cereal fermentation by LAB: from ancient to modern alimentation biotechnologies. In: Ray RC, Paramithiotis S, De Carvalho Azevedo VA, Montet D (eds) *Lactic Acid Bacteria in Food Biotechnology: Innovations and Functional Aspects*. Elsevier, pp 3–26. <https://doi.org/10.1016/B978-0-323-89875-1.00017-1>
9. Tamang JP, Watanabe K, Holzapfel WH (2016) Review: diversity of microorganisms in global fermented foods and beverages. *Frontiers in Microbiology* 7:377. <https://doi.org/10.3389/fmicb.2016.00377>
10. Terpou A, Papadaki A, Lappa IK, Kachrimanidou V, Bosnea LA, Kopsahelis N (2019) Probiotics in food systems: significance and emerging strategies towards improved viability and delivery of enhanced beneficial value. *Nutrients* 11(7):1591. <https://doi.org/10.3390/nu11071591>
11. Tripathi MK, Giri SK (2014) Probiotic functional foods: survival of probiotics during processing and storage. *Journal of Functional Foods* 9:225–241. <https://doi.org/10.1016/j.jff.2014.04.030>
12. Amrane A, Prigent Y (1998) Influence of yeast extract concentration on batch cultures of *Lactobacillus helveticus*: growth and production coupling. *World Journal of Microbiology and Biotechnology* 14(4):529–534. <https://doi.org/10.1023/A:1008828415639>
13. Burgos-Rubio CN, Okos MR, Wankat PC (2000) Kinetic study of the conversion of different substrates to lactic acid using *Lactobacillus bulgaricus*. *Biotechnology Progress* 16(3):305–314. <https://doi.org/10.1021/bp000022p>