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Interesting Starter Culture Strains for Controlled Cocoa Bean Fermentation Revealed by Simulated Cocoa Pulp Fermentations of Cocoa-Specific Lactic Acid Bacteria[▽]

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Among various lactic acid bacterial strains tested, cocoa-specific strains of *Lactobacillus fermentum* were best adapted to the cocoa pulp ecosystem. They fermented glucose to lactic acid and acetic acid, reduced fructose to mannitol, and converted citric acid into lactic acid and 2,3-butanediol.

Fermented dry cocoa beans are the basic raw material for chocolate production. Cocoa beans are the seeds of the cocoa tree, *Theobroma cacao* L. The key microorganisms for successful cocoa bean fermentation processes are yeasts, lactic acid bacteria (LAB), and acetic acid bacteria (AAB) (1, 3–5, 14, 18–21). Although the LAB species diversity involved in the onset of any spontaneous cocoa bean fermentation is wide, not much is known about how cocoa-specific LAB species, such as *Lactobacillus fermentum*, adapt physiologically and what their targeted functional roles are during fermentation (1, 3, 4, 14, 19–21). The present study aimed at the kinetic investigation of carbohydrate fermentation and citric acid conversion by various LAB strains to unravel this.

The LAB strains used throughout this study are listed in Table 1. Monoculture fermentations were performed in 1.5 liters of a cocoa pulp simulation medium (CPSM) for LAB (16) in Biostat B-DCU fermentors (Sartorius AG/B. Braun Biotech International, Melsungen, Germany) anaerobically for 48 h. Inoculum build-up, fermentor setup, online control of temperature (Table 2), pH profile, agitation, and sampling were as described previously (13, 16). All fermentations were performed in duplicate. The results and figures presented hereinafter are representative for both fermentations.

During fermentation, bacterial growth (CFU per ml) was quantified through plating of 10-fold serial dilutions of the samples in saline (0.85% [wt/vol] NaCl solution) on CPSM agar (CPSM containing 1.5% [wt/vol] agar, pH 5.5) that was incubated at the appropriate fermentation temperature for 24 h. Metabolite concentrations were determined through high-performance anion-exchange chromatography using a standard addition protocol (glucose, fructose, mannitol, and citric acid) (15, 23) and high-performance liquid chromatography using external standards (lactic acid, acetic acid, and ethanol) (16). Cell count and metabolite (with external standards) measurements were performed on three independent samples. The errors on the measurements are represented as standard deviations. Gas chromatography (GC) was used for the qualitative determination of 2,3-butanediol and acetoin (15). Concentrations of carbon dioxide in the fermentor gas effluents were determined online through GC (13). The carbon recovery (CR, expressed as percentage) was calculated by dividing the total amount of carbon recovered in the metabolites by the total amount of carbon present in the carbon sources.

All LAB strains tested were able to grow in CPSM (Fig. 1 and Table 2). Strictly heterofermentative LAB strains (all *L. fermentum* and *Weissella* strains, *Leuconostoc pseudomesenteroides* 22, and *Fructobacillus pseudoficulneus* M83) fermented glucose, converted citric acid (not in the case of *Lc. fermentum* IMDO 130101 and *F. pseudoficulneus* M83 and only at the end of the fermentation in the case of *Lc. pseudomesenteroides* 22), and reduced fructose (not in the case of *Weissella ghanensis* LMG P-23179 and *Weissella fabaria* LMG 24289^T). The facultative heterofermentative *Lactobacillus plantarum* 80 (hardly converted citric acid) and *Lactobacillus fabifermentans* LMG 24284^T (did not convert citric acid) strains fermented glucose and fructose simultaneously, with a preference for fructose. The facultative heterofermentative *Lactobacillus cacaonum* LMG 24285^T fermented fructose but not glucose and converted citric acid. Due to the initial low pH (3.5), *Enterococcus casseliflavus* M484 and *Lactobacillus amylovorus* DCE 471 were not able to grow in CPSM.

This study showed that cocoa pulp was actually an ideal substrate for strictly heterofermentative (e.g., *L. fermentum*) and fructophilic LAB species (e.g., *F. pseudoficulneus*), because it contains a high concentration of fructose (energy source and/or alternative external electron acceptor) and citric acid (additional source of pyruvate). These substrates are used for the oxidation of NADH + H^+ to bypass the energy-limiting ethanol pathway and, so, to maximize their growth rate on glucose, thereby producing mannitol and lactic acid plus acetic acid, respectively (16). They are to be consumed under low-pH and anaerobic conditions in the beginning of the cocoa bean fermentation. Citric acid conversion by strictly heterofermentative *L. fermentum* strains seems to be source dependent,

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TABLE 1. Overview of the cocoa-specific and cocoa-nonspecific strains of lactic acid bacteria (LAB) used throughout this study

LAB strain[a]	Source	Reference(s)
Cocoa-specific strains		
E. casseliflavus M484	Brazilian cocoa bean box fermentation	21
F. pseudoficulneus M83	Brazilian cocoa bean box fermentation	21
L. cacaonum LMG 24285^T	Ghanaian cocoa bean heap fermentation	4, 6
L. fabifermentans LMG 24284^T	Ghanaian cocoa bean heap fermentation	4, 6
L. fermentum 222	Ghanaian cocoa bean heap fermentation	3
L. fermentum M103	Brazilian cocoa bean box fermentation	21
L. fermentum M158	Brazilian cocoa bean box fermentation	21
L. fermentum M332	Brazilian cocoa bean box fermentation	21
L. plantarum 80	Ghanaian cocoa bean heap fermentation	3
Lc. pseudomesenteroides 22	Brazilian cocoa bean box fermentation	21
W. fabaria LMG 24289^T	Ghanaian cocoa bean heap fermentation	3, 7
W. ghanensis LMG P-23179	Ghanaian cocoa bean heap fermentation	3, 8
Cocoa-nonspecific strains		
L. amylovorus DCE 471	Corn steep liquor	9
L. fermentum IMDO 130101	Belgian sourdough fermentation	22

[a] LMG, Belgian Coordinated Collections of Microorganisms/Laboratory for Microbiology Ghent (BCCM/LMG; Ghent, Belgium); IMDO, Research Group of Industrial Microbiology and Food Biotechnology (Vrije Universiteit Brussel, Brussels, Belgium); DCE, Department of Chemistry and Engineering (Vrije Universiteit Brussel, Brussels, Belgium).

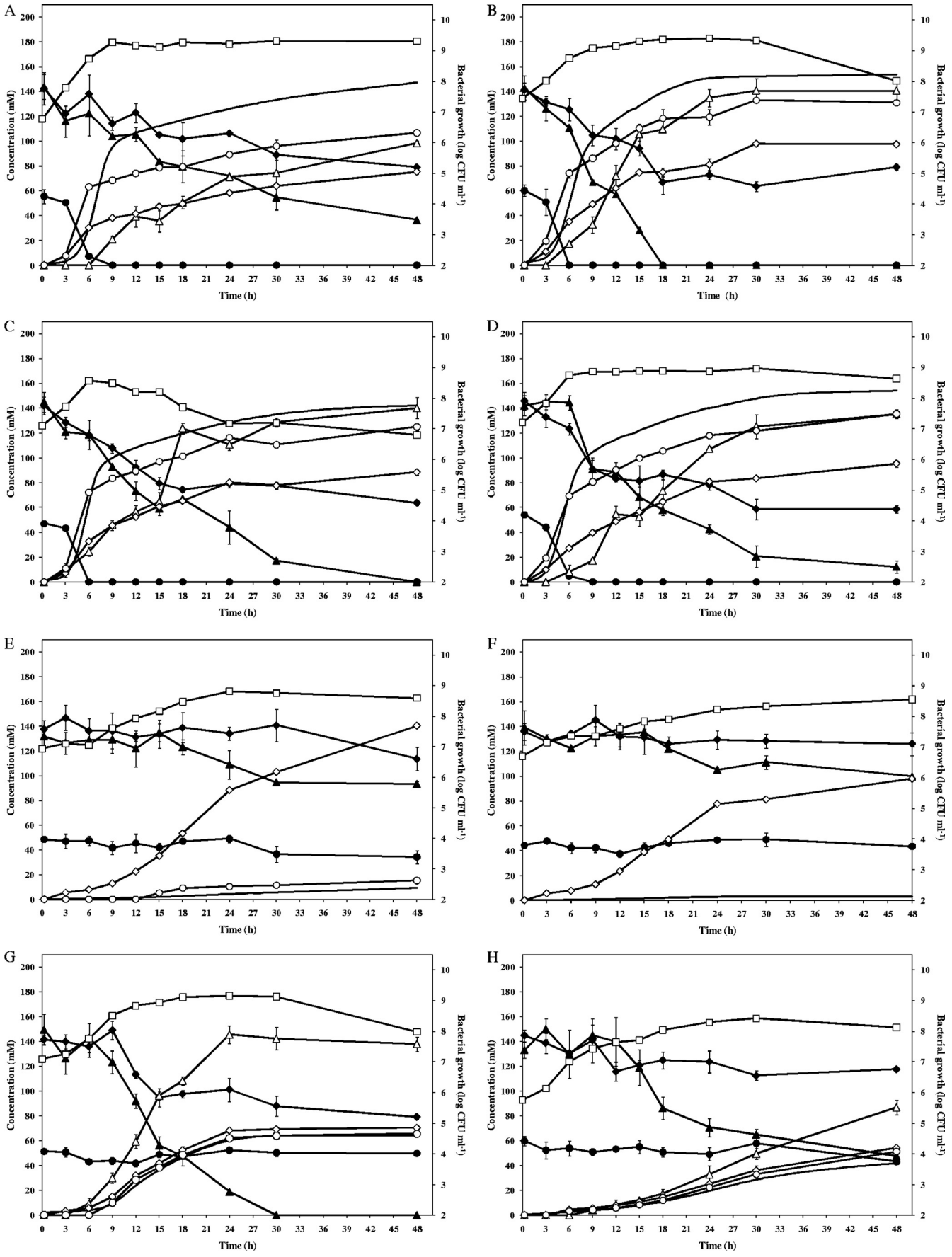
because the sourdough-specific *L. fermentum* IMDO 130101 strain lacked the ability to convert citric acid. As *L. fermentum* is strictly heterofermentative and heat, acid, and ethanol tolerant, it usually dominates successful cocoa bean fermentation processes, independent of the cocoa-producing region (3, 4, 10, 14, 19–21). Also, fructophilic LAB species seemed to be well-adapted to the cocoa pulp ecosystem and, indeed, *F. pseudoficulneus* has been recovered from cocoa bean fermentations (19, 20). They are generally associated with fructose-rich niches and grow on fructose (preferentially) or on glucose in the presence of alternative external electron acceptors (12). Up to now, only strictly heterofermentative LAB species have been reported as fructophilic LAB species. In this study, the facultative heterofermentative *L. plantarum* 80, *L. fabifermentans* LMG 24289^T, and *L. cacaonum* LMG 24284^T strains were characterized as fructose-loving LAB strains also. This indicates their adaptation to the cocoa pulp habitat. These cocoa-specific strains fermented fructose essentially to lactic acid. Hence, citric acid-converting, mannitol-producing (fructose-reducing), heterolactic, and/or fructose-loving LAB strains are particularly adapted to the cocoa pulp matrix. They represent interesting starter cultures to be exploited for enhanced and controlled cocoa bean fermentations.

In the present study, citric acid conversion by cocoa-specific LAB strains led to the production of the butterlike flavor compounds acetoin (*L. cacaonum* LMG 24285^T, due to fructose homolactate fermentation) and 2,3-butanediol (all cocoa-specific *L. fermentum* strains, *W. fabaria* LMG 24289^T, and *W.*

TABLE 2. Carbohydrate and citric acid consumption and metabolite production of cocoa-specific and cocoa-nonspecific LAB strains in a cocoa pulp simulation medium for lactic acid bacteria

Strain	Fermentation temp (°C)	Mean consumption ± SD (mM) of substrate (after 48 h of fermentation)			Mean production ± SD (mM) of metabolites (after 48 h of fermentation)					Carbon recovery (%)
		Glucose	Fructose	Citric acid	Lactic acid	Acetic acid	Carbon dioxide	Mannitol	Flavor compound[a]	
L. fermentum 222	37	63.4 ± 13.1	107.2 ± 10.7	55.4 ± 5.6	75.3 ± 1.8	106.7 ± 2.0	147.1 ± 0.1	98.3 ± 2.0	22	93
L. fermentum M103	37	62.1 ± 8.1	142.9 ± 9.6	60.2 ± 4.4	97.5 ± 1.2	131.0 ± 1.3	153.8 ± 0.1	140.6 ± 9.6	16	102
L. fermentum M158	37	78.1 ± 9.6	145.0 ± 8.1	46.9 ± 0.0	88.4 ± 1.9	125.0 ± 2.7	142.1 ± 0.1	140.0 ± 8.5	18	97
L. fermentum M332	37	87.2 ± 10.1	130.1 ± 12.9	54.1 ± 1.5	95.3 ± 2.4	135.5 ± 3.8	154.2 ± 0.1	135.1 ± 0.3	20	98
L. plantarum 80	37	24.2 ± 16.2	38.3 ± 10.0	14.3 ± 6.9	140.3 ± 0.6	15.2 ± 0.5	9.2 ± 0.1	0	0	100
L. fabifermentans LMG 24284^T	30	9.9 ± 15.9	39.3 ± 13.9	0	98.0 ± 1.4	0	0	0	0	101
L. fermentum IMDO 130101	37	62.5 ± 5.3	143.7 ± 7.9	0	70.2 ± 1.1	65.5 ± 1.2	64.2 ± 0.1	138.0 ± 5.7	0	100
Lc. pseudomesenteroides 22	30	27.0 ± 5.6	85.5 ± 7.3	16.6 ± 4.5	54.1 ± 1.2	51.2 ± 1.2	41.7 ± 0.1	82.8 ± 6.0	0	104
F. pseudoficulneus M83	28	52.4 ± 15.5	81.7 ± 19.5	0	50.4 ± 0.2	46.9 ± 0.3	44.2 ± 0.1	89.0 ± 6.9	0	102
W. ghanensis LMG P-23179	30	2.9 ± 7.5	0	52.7 ± 3.3	10.0 ± 0.6	57.8 ± 5.0	111.1 ± 0.1	0	26	108
W. fabaria LMG 24289^T	30	21.7 ± 17.1	0	46.6 ± 1.2	18.7 ± 0.6	66.0 ± 1.6	108.2 ± 0.1	0	24	96
L. cacaonum LMG 24285^T	30	0	28.3 ± 24.5	47.7 ± 2.1	63.4 ± 0.4	51.4 ± 1.2	88.1 ± 0.1	0	20	101

[a] Theoretical, estimated flavor compound production by the cocoa-specific LAB strains, according to the pathway proposed by Mayo et al. (17). In all cases, the flavor compound produced was 2,3-butanediol, except for the *L. cacaonum* LMG 24285^T monoculture fermentations, where the flavor compound was acetoin.


A
B
C
D
E
F
G
H
Concentration (mM)
Bacterial growth (log CFU ml-1)
Time (h)

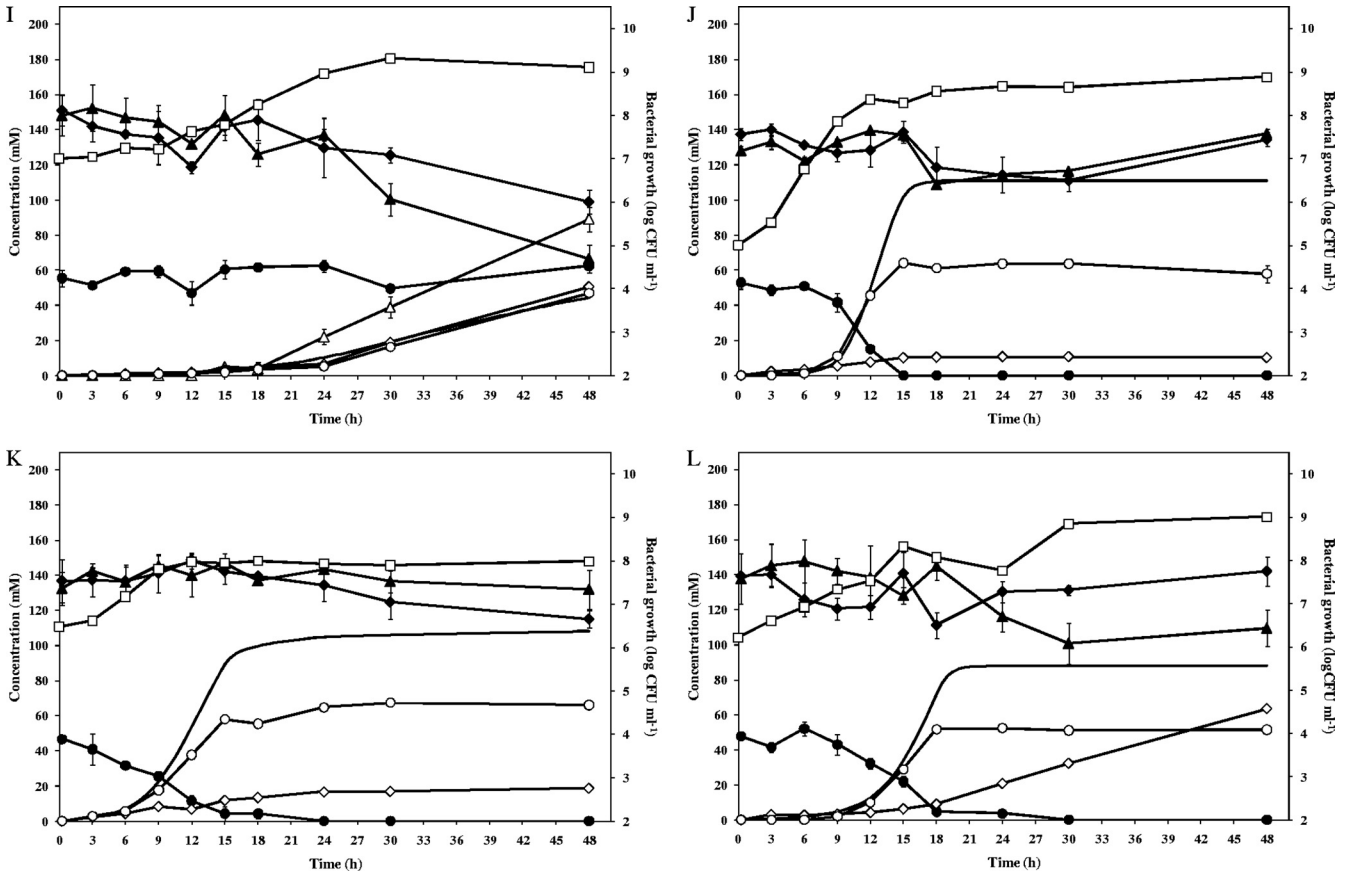


FIG. 1. Bacterial growth, carbohydrate and citric acid consumption, and metabolite production of *Lactobacillus fermentum* 222 (A), *Lactobacillus fermentum* M103 (B), *Lactobacillus fermentum* M158 (C), *Lactobacillus fermentum* M332 (D), *Lactobacillus plantarum* 80 (E), *Lactobacillus fabifermentans* LMG 24284^T (F), *Lactobacillus fermentum* IMDO 130101 (G), *Leuconostoc pseudomesenteroides* 22 (H), *Fructobacillus pseudoficulneus* M83 (I), *Weissella ghanensis* LMG P-23179 (J), *Weissella fabaria* LMG 24289^T (K), and *Lactobacillus cacaonum* LMG 24285^T (L) in a cocoa pulp simulation medium for lactic acid bacteria. Glucose, ◆; fructose, ▲; citric acid, ●; lactic acid, ◇; acetic acid, ○; mannitol, △; carbon dioxide, —; and bacterial growth, □.

ghanensis LMG P-23179, due to the need for extra NAD^+ recuperation) (17). These compounds form part of the flavor profile of certain cocoa-based products (2, 11).

LAB species are important for a successful microbial succession during cocoa bean fermentations. Actually, LAB form the link between the ethanol- and flavor-producing yeast fermentation and the acetic acid-producing AAB fermentation (10, 16). The lactic acid and mannitol they produce could serve as extra energy sources for AAB species, while their citric acid conversion results in a rise in pH and a possible contribution to cocoa flavor. So, LAB strains, either as monoculture or coculture, will be essential components of starter cultures aimed at the control of cocoa bean fermentation processes to obtain well-fermented dry cocoa beans and improved standard- and superior-tasting chocolates produced therefrom.

In summary, the kinetics of both aqueous and gaseous metabolite production by cocoa-specific and cocoa-nonspecific LAB strains revealed a deeper insight into their energy and citric acid metabolism and metabolite production patterns. The full meaning of these results for the actual control of cocoa bean fermentations by the use of these strains as appropriate starter cultures is under investigation. Nevertheless, this kinetic study contributes to a better understanding of the functional behavior of cocoa-specific LAB strains to be used as interesting starter cultures for controlled cocoa bean fermentations. In addition, the cocoa-specific *L. fermentum* strains can be categorized as the ones best adapted to the cocoa pulp ecosystem and show interesting functional roles for the development of a defined starter culture.

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