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Screening of Lactic Acid Bacteria Capable to Breakdown Citric Acid during Ivorian Cocoa Fermentation and Response of Bacterial Strains to Fermentative Conditions

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Authors' contributions

This work was carried out in collaboration between all authors. Authors SLN and HGO designed the study. Authors SLN, HGO and JNA draft the manuscript. Authors HGO, DHO and BGG: wrote the protocol. Authors DHO, BGG, GAK and GGD managed the literature searches and performed the analysis. All authors read and approved the final manuscript.

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ABSTRACT

Aims: The breakdown of citric acid contained in the pulp during cocoa fermentation is an important and key property for bacterial growth and for obtaining a well fermented cocoa. The objective of this study was to analyze citrate metabolism in lactic acid bacteria (LAB) isolated from Ivorian fermenting cocoa beans and evaluate their capacity to grow effectively under fermentation conditions.

Place and Duration of Study: Laboratory of Biotechnology, UFR Biosciences, University Félix Houphouet-Boigny (Côte d'Ivoire), between August 2014 and April 2015.

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Methodology: Spontaneous heap fermentations were conducted in three cocoa producing regions during 6 days. Bacteria isolation was performed using plate culture on MRS medium and strains were screened for citrate metabolism using Kempler and McKay medium whereas gas and acetoin productions from citrate were searched. Additionally, the viability of cells under stress conditions related to cocoa fermentation was tested.

Results: The results show that a wide rate of LAB strains (75%), mainly heterofermentative possess citrate metabolism, and most of these strains produce gas from citrate but were not able to produce acetoin from citrate. Moreover, some LAB presenting citrate metabolism show a remarkable thermotolerance at 45°C with more than 50% of survival growth rate (SGR), while some exhibited a poor viability (less than 10%) at this temperature. Ethanol at 8-12% was found to have no adverse effect on bacterial growth. In contrast, lactic acid, acetic acid and citric acid exerted individually full inhibition on LAB strains that failed to grow at 0.4% of acid.

Conclusion: Taken together, the results indicate that strains studied may preferentially produce lactic acid from citrate and their high proportion should contribute to efficiently break down citric acid during cocoa fermentation. However, occurrence of a high acidity could seriously limit the growth of these valuable potential starter strains in fermentation conditions.

Keywords: Lactic acid bacteria; citrate metabolism; cocoa fermentation; screening starter; Côte d'Ivoire.

1. INTRODUCTION

Fermentation is the first step of cocoa processing for chocolate production. It is led by a complex microbial consortium essentially composed of yeasts, lactic acid bacteria (LAB), acetic acid bacteria (AAB) and *Bacillus* which impact strongly the quality of cocoa and chocolate [1,2]. During cocoa fermentation, yeasts oxidize sugar contained in the pulp, into ethanol that is further oxidized into acetic acid by acetic acid bacteria. This simple chain of reactions lead to a raise of temperature in fermenting cocoa mass, and combined with the acidity produced, allows activation of hydrolytic enzymes deep into cotyledon [3]. Consequently, the specific precursors of chocolate aroma and flavor are generated in the cotyledon as a results of triggering effect of microbial activity [4-6].

The competition due to the complex and numerous microorganisms involved in cocoa fermentation lead to a succession of microbial growth depending on environment conditions changes during the process. It is clearly established that fermentation beginning in anaerobic and high sugar content conditions, allows first, the growth of yeasts [1]. As culture conditions are changing in the fermenting mass, notably the oxygen pressure, temperature and pH, lactic acid bacteria grow at 48 h, followed by acetic acid bacteria 72 h and *Bacillus* 84-108 h [7,8].

One of the most important conditions which strongly impact the microbial growth during cocoa fermentation is the pH. In fact, fresh cocoa

pulp before fermentation, is acidic with a pH round 3.5 due to the presence of citric acid [1]. This acidic pH, although allowing fungal growth, is not favorable for bacterial propagation. Hence, an effective and proper course of cocoa fermentation is characterized by a progressive increase of the pH that could reach 5 to 8, at the end of fermentation [9-11]. Therefore, the raise of pH is fundamental for bacterial growth resulting in many biochemical reactions, which lead to the production of chocolate with high quality [12]. Additionally, the increase of pH is essentially known as a result of the breakdown of citric acid contained in the cocoa pulp [1]. Citrate metabolism become therefore a key and relevant property desirable in microflora to achieve a good fermentation [13,14]. Yeasts and more importantly lactic acid bacteria are suggested to be responsible for the breakdown of citric acid during cocoa fermentation [1,15].

However, citrate is consumed in lactic acid bacteria to produce various compounds such as succinic acid, lactic acid, acetoin or butane 2, 3 diol, depending on the metabolic pathways [14]. Additionally, the efficiency of a microorganism to breakdown citric acid is related to the metabolic pathway used. For instance, heterofermentative strains of LAB involved in cocoa fermentation, are susceptible to metabolize citrate more efficiently than homofermentative [9]. Yet, the screening of strains presenting an efficient citrate metabolism is important to target the most valuable as starter in the context of control and standardization of cocoa fermentation process [16,15,11]. Indeed, the quality of fermented and dried cocoa bean produced from spontaneous

and natural cocoa fermentation remains of variable quality leading very often to low crop value for farmers.

Previously, in a preliminary study, we screened a few LAB strains from cocoa fermentation, showing citrate metabolism but with no further analysis [10].

In this paper, we analyzed the ability of LAB involved in Ivorian cocoa fermentation to break down citric acid and evaluated the capacity of screened strains, to grow effectively under cocoa fermentation conditions.

2. MATERIALS AND METHODS

2.1 Fermentation Conditions

Cocoa pods were harvested from traditional farms in three producing regions in Côte d'Ivoire notably Agnéby-Tiassa (geographic coordinates 5°59' North 4°28' West), Lôh-Djiboua (5°55' North 5°37' West) and Sud-Comoé (5°28'06' North 3°12'25' West). Pods were broken, opened manually and beans were extracted, pooled and heap fermented for six days on banana leaves. During fermentation, 200 g of samples were withdrawn and transferred into sterile plastic bag (stomacher) at 12 h intervals for analysis purpose, whiles at the same time, temperature and pH were recorded directly on the 100 g fermenting mass.

2.2 Isolation of Bacterial Strains

Samples were brought to laboratory and subjected to microbial analysis. From these samples, 25 g of fermenting cocoa beans were mixed in 225 mL of 0.1% (w/v) peptone water (Oxoid, Basingstoke, United Kingdom) and shaken vigorously to obtain a uniform homogenate. An aliquot (1.0 mL) of the homogenate was serially diluted 10-fold in saline Trypton buffer and plated onto different selective agar media containing 50 µ/mL of nystatin as fungal growth inhibitor [10]. Plates were incubated at 37°C for 48-72 h under jar anaerobic conditions. Lactic acid bacteria associated with fermenting cocoa was isolated as Gram positive, catalase and oxidase negative. Isolates were stored at -80°C in MRS buffer medium supplemented with 20% (v/v) glycerol in Eppendorf tubes, for further studies.

2.3 Screening of Bacteria for Citrate Metabolism

The capability of lactic acid bacteria to metabolize citrate was investigated using the method described by Kempler and McKay [17]. A basal medium containing 1% milk powder, 0.25% casein peptone, 0.5% glucose and 1.5% agar was first prepared and then 1 L of the medium, was supplemented with 10 mL of potassium ferricyanide (10%) solution and 10 mL of mixed iron citrate and sodium citrate (2.5%) solution. The medium was inoculated with pure 24 h pre-culture of LAB strains and incubated at 30°C for 48-72 h. The citrate metabolism is assessed by the formation of a blue complex on the colony resulting in the citrate consumption.

Furthermore, as confirmation tests, the secondary product of citrate metabolism such as gas (CO₂) and acetoin production from citrate as sole carbon source were searched. To monitor gas production, a volume of 10 mL of the basal medium with sodium citrate as sole carbon source, contained in a 20 mL tube was seeded with LAB strains by a central sting touching the bottom of the tube. Gas production is visible in the tube after incubation.

Acetoin production from citrate was checked in the basal liquid medium. After 48 h culture incubation, acetoin production was revealed in 1 mL of cell free supernatant by adding two drops of an alcoholic solution of alpha-naphthol 6% and two drops of sodium hydroxide 16% solution (VP). The presence of acetoin is assessed by appearance of red color.

2.4 Growth of LAB under Alcohol and Acid Conditions

Cocoa fermentation conditions result in addition of several single factors such as alcohol, acid and sugar content in the pulp as well as temperature and pH that influence the microbial growth [18,19].

To analyze the growth of LAB in alcoholic and acidic conditions a MRS solid medium was prepared. After autoclaving, this medium is cooled and maintained in liquid state at 45°C and supplemented with alcohol or acid at final concentration ranging from 0.1 to 15% depending on the compound. The standard inoculum was prepared as follow: A 24h pre-culture was suspended in a saline solution to obtain an absorbance OD₆₀₀= 0.1, and this

suspension was used to make a serial decimal dilution up to 10^{-5} . Finally 0.1 mL of this dilution was used to inoculate the different plate media. Hence, unless otherwise specified, the same standard load of bacterial strains was used for all study. After 48-72 h incubation the survival growth rate was evaluated by enumeration of colony and calculation using the following formula: Survival rate = $(\sum \text{yeast colony with alcohol or acid} / \sum \text{yeast colony in negative control}) \times 100$ [20].

2.5 Effect of Temperature and pH on LAB Growth

The growth of bacterial strains in various conditions of temperature and pH was estimated using the standard inoculum described above. The bacterial load was plated onto a MRS medium and cultures were incubated at different temperatures ranging between 30 and 50°C, at 5°C intervals.

Effect of pH on bacterial growth, was estimated in MRS liquid medium by incubating the cultures in different pH conditions.

3. RESULTS AND DISCUSSION

3.1 Citrate Metabolism of LAB Isolates

Lactic acid bacteria associated with cocoa fermentation in three Ivorian regions were isolated and screened for their ability to metabolize citrate.

The results show that the number of LAB strains isolated was variable with the region. For instance, the LAB flora isolated from fermenting cocoa in Lôh-Djiboua was approximately two-folds more important than that isolated from fermenting cocoa in Sud Comoé (Table 1). This variability is not surprising since it is well known that the distribution of the microflora involved in cocoa fermentation is not uniform from a region to another [21-23]. However, the impact of a given group of microorganism on cocoa fermentative process should not necessary be

linked to its load. Most importantly, the type of metabolic pathway susceptible to play a key role in the biochemical course of the fermentative process should be determinant [14].

To this point of view, citrate metabolism stands an important and relevant property desirable for a proper and fine cocoa fermentation [1,15,14] and then strains exhibiting this property are potentially interesting as starter.

The screening of LAB flora isolated, revealed that an important proportion presented citrate metabolism (Table 1). The results show that more than 75% of LAB flora involved in Lôh-Djiboua and Sud-Comoé cocoa fermentation have the property to break down citric acid as revealed by color change of the colonies (Fig. 1) while this proportion remains relatively high (68.85) among the LAB population of Agnéby-Tiassa. At date, no study has been reported on the rate of LAB strains capable to consume citric acid during cocoa fermentation. This study clearly indicates that all LAB flora do not contribute to citric acid consumption during Ivorian cocoa fermentation although the breakdown of this acid is probably achieved by a cooperation of a wide population of LAB. As citrate metabolism is an essential factor for modulating the pH and promoting the growth of desirable bacterial flora in fermenting cocoa [13,14] the high rate of bacterial population capable of metabolizing citric acid observed in this study should be widely responsible for the raise of pH recorded during fermentation of cocoa in these regions (Fig. 2).

It is known that citric acid imparts to the beans an initial pH of 3.5 to 4.5 before fermentation. In this study, it was observed a sharp raise of pH after 48h of fermentation in the three regions (Fig. 2), corresponding to effective growth of LAB during this period of fermentation (data not shown). The high proportion of LAB strains that consume citric acid observed in this study, should be determinant most importantly, for a well processed fermentation and the quality of products.

Table 1. Distribution of lactic acid bacteria isolated from fermenting cocoa in different regions

Regions	Total isolates	Citrate positive isolates		
		Homofermentative	Heterofermentative	Total
Lôh-Djiboua	210	48	112	160 (76.19 %)
Sud-Comoé	114	37	57	94 (82.45 %)
Agnéby-Tiassa	183	61	65	126 (68.85 %)

The numerals express the number of strains and number in parenthesis express the percentage

The method described by Kempler et McKay 1980 was used. White colonies: Bacterial strains unable to metabolize citrate. Blue colonies: bacterial strains capable to break down citrate.

We further analyzed the citrate metabolism pathway in our LAB isolates in order to point out which metabolic pathway is preferentially used for citrate consumption in these strains during cocoa fermentation. For this purpose, gas and acetoin production using citrate as sole carbon source were checked. The results showed that, a proportion of LAB strains comprised between 45 and 60% depending on the region, were able to produce gas from citrate metabolism, whereas no strain was able to produce acetoin from citrate (Table 2). To understand the significance of these results we retrieved from metagenomic data generated by previous study [14] all data concerning citrate metabolism that have been resumed in Fig. 3. According to these data (Fig. 3) citrate could be metabolized in LAB from cocoa fermentation via two major pathways that we named pI and pII (Fig. 3). pI produces succinate from citrate but produces no gas, while pII produces acetoin and gas from citrate Fig 3. In our study, we can classify LAB isolates unable to produce gas from citrate as pI pathway probable users responsible for succinic acid production. This is supported by previous study that reported the presence of succinic acid in fermenting cocoa [19], suggesting that this acid may in part come from citric acid metabolism from LAB using pI pathway.



Fig. 1. Screening of lactic acid bacteria presenting citrate metabolism

The other portion of LAB strains isolated in this study, producing gas from citrate can be classified as using pII metabolic pathway responsible for acetoin or diacetal production (Fig. 3). Unfortunately all our LAB strains isolated

from fermenting cocoa failed to produce acetoin from citrate. In contrast, acetoin or diacetal production from citrate have been generally observed in lactic acid bacteria from samples other than cocoa. Our results suggest that LAB isolated from cocoa, do not use entirely pII metabolic pathway, turning preferentially to lactic acid production (Fig. 3). Previous study reported that a heterofermentative strain *Lactobacillus fermentum*, considered as best adapted to cocoa pulp ecosystem and interesting starter, isolated from Ghanaian fermenting cocoa, was capable to metabolize citrate into lactic acid [15]. Hence, it could be assumed that strains capable to produce gas from citrate and unable to produce acetoin, analyzed in this study are potentially lactic acid producers and then this type of strains might play an interesting role for a well-processed fermentation of cocoa. Interestingly, this type of strains were also found to have the heterofermentative feature in a large proportion (Table 2). Recent studies reported that the conversion of citric acid into lactic acid by heterofermentative strains was more efficient than the conversion of carbohydrates such as glucose and fructose into this compound [9]. This strongly supports the fact that our LAB strains heterofermentative, capable to produce gas from citrate and unable to produce acetoin may be responsible for a rapid and active consumption of citric acid and may help to speed the fermentation process.

Table 2. Distribution of lactic acid bacteria responding to different tests of citrate metabolism

	Lôh-Djiboua	Sud-Comoé	Agnéby-Tiassa
Blue colony	160	94	126
Gas from citrate	101	46	33
Acetoin from citrate	0	0	0

3.2 Effect of Temperature on LAB Citrate Positive

Lactic acid bacteria producing gas from citrate and presenting heterofermentative feature, were further analyzed for their growth under stress conditions; five strains from different cocoa producing regions, were used as models. Fig. 4, shows two group of strains regarding their grow ability under thermic stress. The first group (I) of LAB strains notably the strains T1D1 and TOD8 presented an excellent capacity to grow at

temperature in 30-45°C range with a maximal growth rate (100%) at 40°C. At 45°C, these two strains give relatively a good response to thermic stress with 48 to 65% of survival growth rate (Fig. 4). The second group (II) of LAB strains presented a less important response to thermic stress. At 40°C, the strains of this group

presented a survival growth rate only ranging between 65 and 76% and nearly failed to grow at 45°C with a residual growth rate of approximately 15%. However, all the strains tested did not support a temperature of 50°C, keeping less than 6% of survival growth rate at this temperature.

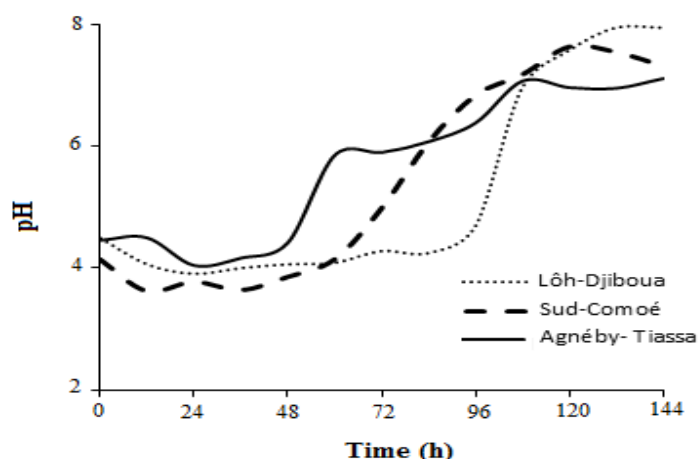


Fig. 2. Evolution of pH in cocoa pulp during fermentation

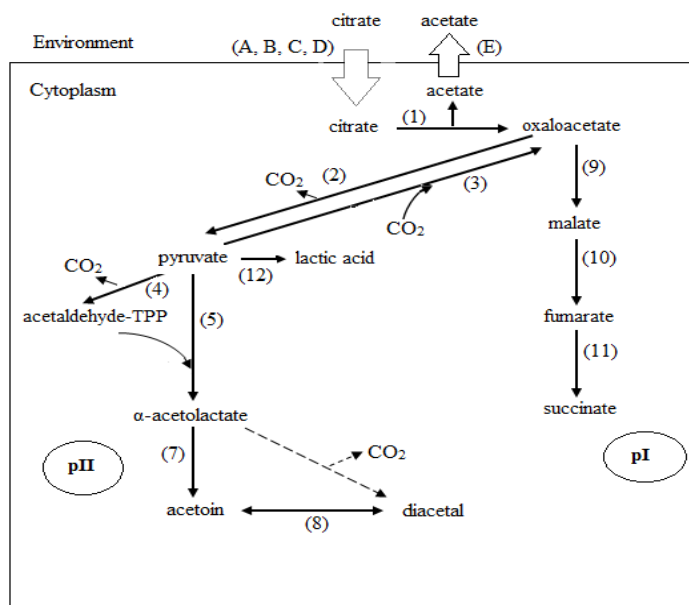


Fig. 3. Citrate metabolism pathways in LAB strains involved in cocoa fermentation [14]

A: uncharacterized di/tricarboxylate transporter; B: GntP-family gluconate, Hb-family symporter (*gntP*); C: CitMHSfamily, citrate transporter (*citM*); A and E: 2HCT-family citrate/acetate antiporter (*citW*); (1) citrate lyase (*citGXFEDC*); (2) NAD⁺ dependent oxaloacetate decarboxylating malate dehydrogenase (*sicA*); (3) pyruvate carboxylase (*pyc*); (4) TPP-dependent pyruvate decarboxylase; (5) α -acetolactate synthetase (*ilvHI*); (7) α -acetolactate decarboxylase (*budA/aldB*); (8) acetoin reductases (*budC/butA*); (9) NAD⁺/FAD-dependent malate dehydrogenase (*mdh*); (10) fumarase (*fumA/fumB/fumC*); (11) fumarate reductases (*sdhABCD*); (12) lactate dehydrogenase (*ldh*)

In general, cocoa in fermentation reached a maximum temperature of 45°C in the Ivorian three regions studied (data not shown). Although, this temperature is believed to be a factor susceptible to limit the expression of microbial potentialities during cocoa fermentation, the strains belonging to the first group of this study (T1D1 and T0D8) displaying a more important capacity of viability at 45°C, should be the principal actors among LAB breaking down citric acid during cocoa fermentation and should be preferentially targeted as starter. Recently, a majority of lactic acid bacteria isolated from cocoa fermentation in Mexico were found to have ability to grow at 45°C [9], among them, a strain of *Lactobacillus fermentum* was selected to be included in a cocktail of starter culture to control cocoa fermentation. The thermotolerance exhibited by some LAB strains capable to metabolize citrate should allow them to remain active under thermic stress conditions during cocoa fermentation.

3.3 Effect of Acids and Alcohol on Bacterial Growth

Fig. 5, shows different responses of strains under acid and alcohol conditions, although all the strains displayed generally maximum growth at 0.1% of acid. Here, it was also observed two groups of strains concerning their ability to grow in acids tested (Fig. 5ABC). The first group of bacterial strains failed to grow at 0.4% of acid while the second group grew with more than 50%

of survival rate at this acid concentration (Fig. 5 ABC). The strain T0D8 described above as the most thermotolerant, was remarkably present among all the group of bacteria presenting the best response to acid stress. This evidences that some strains are more valuable than others and then a screening is necessary to target the best suited for fermentation control. On the other hand, the results show a notable ability of all strains tested, to grow at 8% of ethanol concentration, some strains such as T12D32 and T1D1 being able to grow even at 12% of alcohol with a survival rate round 75% (Fig. 5D). Lactic acid bacteria from Mexican fermenting cocoa were also found to grow at 12% of alcohol concentration [9].

During cocoa fermentation, the concentrations of acid in the pulp are reported to be comprised in the range 0.6-2.5% for acetic acid, 0.2-0.5% for lactic acid and a maximum of 0.3% for citric acid, while ethanol concentration is assumed to be between 0.8 and 2.5% [7,23]. In these conditions, alcohol yielded in cocoa mass during fermentation may not exert an adverse effect on LAB growth and may not hinder citrate metabolism in these strains. However, acids at a threshold concentration of 0.5%, appeared to inhibit fully LAB citrate positive growth and may constitute an important factor that probably prevent the expression of LAB properties in the fermenting mass. Lefeber et al. [13] have also reported a less acid tolerant heterolactic *L. fermentum* strains able to breakdown citrate.

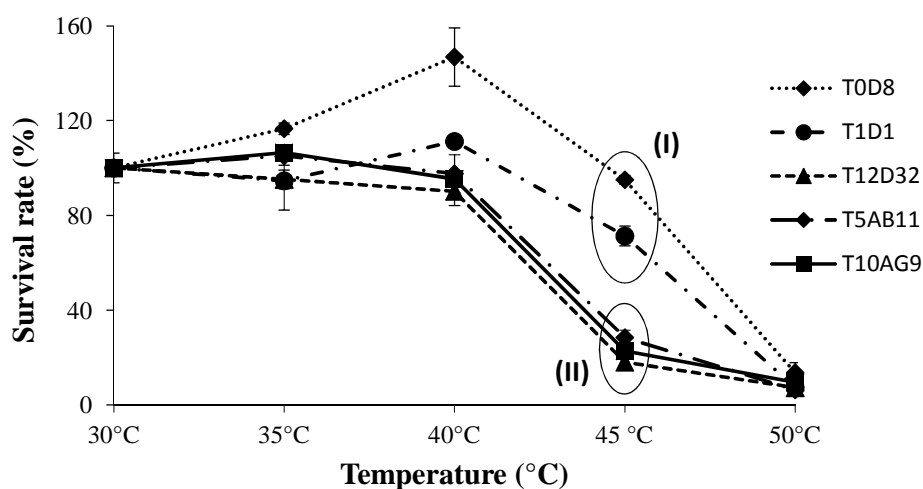


Fig. 4. Effect of temperature on the growth of LAB citrate positive strains
 (I) group of strains presenting remarkable tolerance at 45°C. (II) group of strains less tolerant at 45°C

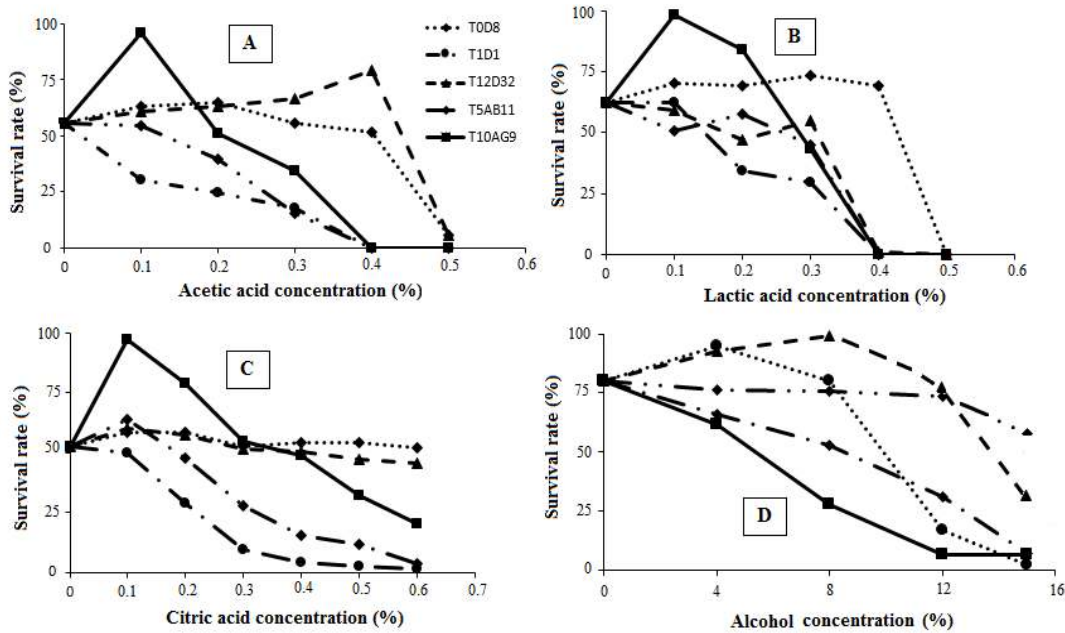


Fig. 5. Effects of acids and alcohol on the growth of LAB citrate positive strains

The competitiveness of these strains should depend on fermentation conditions such as sugar content in the pulp. In fact, a high concentration of glucose and fructose in the pulp leads to a high production of acid [24] which is not suitable for viability of these strains as indicated by our results. In contrast, cocoa pulp preconditioned before fermentation is susceptible to reduce significantly acid yield in fermenting pulp [24] and should be more adequate for optimal expression of citrate metabolism in these strains studied.

Cultures were performed using MRS medium supplemented with the corresponding compound at different concentrations, and incubated at 30°C for 72. Survival growth rate was evaluated by enumeration of colony and calculation using the following formula: Survival rate = $(\sum \text{yeast colony with alcohol or acid} / \sum \text{yeast colony in negative control}) \times 100$ [20].

4. CONCLUSION

A wide proportion of LAB involved in fermenting cocoa in the three regions studied are able to metabolize citrate. This metabolism is characterized by gas production from citrate with no acetoin production. These strains presenting heterofermentative feature use probably the pathway to lactic acid production. This type of strains are efficient in citric acid breakdown. However, the growth and the efficiency of these

strains in fermenting cocoa may strongly depend on acids concentration which exerts full inhibition on bacterial strains at 0.5% concentration.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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