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# Occurrence of High Acetic Acid-Producing Bacteria in Ivorian Cocoa Fermentation and Analysis of Their Response to Fermentative Stress

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**Abstract:** Acetic acid produced during cocoa fermentation impact strongly the quality of fermented and dried cocoa beans and chocolate. The objective of this study was to analyze acid production in Acetic acid bacteria (AAB) and their response to fermentative conditions. AAB strains were isolated from cocoa fermentation and screened for acid production on both solid and liquid media. From 444 isolates, 15 strains yielded 2 to 38.1 g/L of acid in liquid medium. The best acid producers were identified by biochemical typing as *Acetobactersp, Acetobacteraceti*, *A. peroxydans*, *A. pasteurianus* and *Gluconobacteroxydans*. Most AAB strains showed strong tolerance to alcohol with round 45% of survival growth rate (SGR) under 12 % ethanol. AAB strains growing well at 2 % acetic acid (SGRround 47 %), failed to grow at 3 % of this acid. Lactic acid has the most hindering effect on AAB growth, provoking a drop of SGR from 100 % to less than 25 % at only 1 % of lactic acid. Maximum growth of acidifying AAB strains occurred at 35 °C. This study evidences the diversity of technological performances of AAB involved in Ivorian cocoa fermentation and allows targeting the most valuable strains as starters.

**Keywords:** AAB Growth, Acetic Acid Production, *Acetobacter,* Cocoa Fermentation, Stress Tolerance

## 1. Introduction

Fermentation is an indispensable step in the transformation process of cocoa into chocolate and has a great impact on the flavor, color and aroma of cocoa products [1, 2, 3]. During cocoa fermentation, complex biochemical reactions triggered by the microbial flora occur and allow the production of cocoa and chocolate with desirable organoleptic characteristic [4, 2, 5, 6].

Although, the whole microflora responsible for cocoa fermentation including essentially yeasts, lactic acid bacteria, acetic acid bacteria and Bacillus is relatively well-known, their different physiological roles remain complex and not sufficiently elucidated [7, 8, 9, 10, 11].

However, yeasts and acetic acid bacteria present the most well-known roles. Yeast present at the onset of the fermentative process, oxidize sugars contained in the pulp into alcohol and breakdown the pulp by pectinolytic enzymes production [12, 2, 13, 3]. Acetic acid bacteria further oxidize alcohol produced by yeasts, into acetic acid which diffuses deep into cotyledons, acidifying the beans and activating inner hydrolytic enzymes [1, 12, 9, 14]. Furthermore, enzymes activated under acidic conditions notably aspartic endoprotease and serine carboxypeptidase both being pH dependent, generate the specific precursors of chocolate aroma [1, 15]. Hence, the lowering of the pH into cocoa bean is of great importance in the fermentative process for the generation of quality of products.At date, it is well established that acetic acid bacteria play a key role in the formation of the precursors of chocolate flavor [16, 2, 17]. Due to its acidifying properties and the easy diffusion of acetic acid into beans, Acetic acid bacteria are assumed to be one of the most valuable microbial flora in cocoa fermentation [2, 14].

On the other hand, cocoa fermentation is still a traditional process difficult to control which gives variable and non-

reproducible quality of fermented and dried beans leading very often to a low crop value for farmers. As a solution to overcome this problem, the utilization of microbial starter culture stands the most promising approach, since it should permit, to efficiently control and standardize the process of cocoa fermentation [7, 18, 5].

As it is well known that microbial strains with acidification capacity are desirable for the production of cocoa bean and chocolate of quality, the acidification capacity is therefore an interesting technological property to be considered for microbial starter screening. However, no further investigation has been undertaken on this property in these bacterial strains involved in cocoa fermentation.

This paper report the biochemical characterization of AAB strains presenting high acidification capacity and evaluation of their growth ability under fermentative stress conditions.

# 2. Material and Methods

### *2.1. Fermentation Conditions and Isolation of Acetic Acid Bacteria*

Cocoa pods were collected from three areas of Côte d'Ivoire notably Agboville (geographic coordinates 5°59' North 4°28'West), Divo (5°55' North 5°37'West) and Aboisso (5°28'06' North 3°12'25' West). Fermentation was processed for six days as described by Yao et al. [19]. Samples were withdrawn at 12 h intervals for acetic acid bacteria isolation and numeration. After appropriate dilution in sterile saline solution, 0.1 mL of the fresh fermenting samples were plated on potato medium containing 0.5 % D-glucose, 1 % yeasts extract, 1 % peptone, 2 % glycerol, 1.5 % potato and 4 % ethanol (v/v), supplemented with 0.0016 % bromocresol green to monitor pH variation and nystatin (50 µg/ml) to inhibit fungal growth[20]. After 48 h incubation at 30 °C, Acetic acid bacteria were identified based on following tests: Gram staining, catalase, oxidase and respiratory metabolism. Colony count enumeration was expressed as CFU per gram cocoa pulp-bean mass. The strains isolated were kept at -80 °C in Luria Bertani medium supplemented with glycerol 20 % in Eppendorf tubes, for further studies.

### *2.2. Quality Control Methods*

#### *2.2.1. Screening of Acidifying AAB Strains*

Screening of acidifying AAB isolates was carried out in both solid and liquid media.

In solid medium the strains were screened for their acidifying property based on clear halo diameter using Hestrin-Schramm (HS) medium composed of 0.05 % glucose, 0.3 % peptone, 0.5 % Yeast extract, 1.5 % $CaCO_3$, 1.2 % agar, and 4 % ethanol as describedinprevious study [21]. The medium was spot inoculated with pure 24 h pre-culture of bacterial strain and incubated at 30 °C for 10 days. Acidifying capability of strains was assessed by acid forming colony characterized by a clear halo with a diameter related to the amount of acid produced.

In liquid medium, the production of acetic acid was evaluated according to the method described by Nanda et al. [22].Acid production medium was prepared by adding 1.0 mL of a 24 hours pre-culture ($OD_{600}$ = 0.5) of acetic acid bacteria strains into 150 mL of calcium free Hestrin-Schramm (HS) broth contained in 250 mL Erlenmeyer flask. To this HS medium, 0.01 % $MgSO_4$ and 0.27 % $NaHPO_4$ were added to promote acid production. The medium was incubated for 10 days under aerobic atmosphere at 30 °C with stirring at 130 rpm. During incubation a 10 mL daily sample of liquid medium were taken and analyzed for determination of growth rate, pH of medium and quantification of acid produced.

The growth rate was directly determinate by measurement of culture medium absorbance at 600 nm. The pH was measured in cell free supernatant after centrifugation of the culture medium at 4500 rpm. Acid yield was quantified in cell free supernatants by titration with NaOH (0.1 N) and phenolphthalein as a color indicator. Acid production was expressed as grams of acetic acid per 100 mL using the following relation:

$$\text{Acidity (\%)} = [V_{(NaOH)} \times 0.1 \times 0.06 / V_{(\text{supernatant used})}] \times 100$$

#### *2.2.2. Characterization of High Acetic Acid Producing Strains*

AAB displaying high acid production were subjected to biochemical characterization for further identification of strains at species level. A scheme of biochemical tests allowing a simple and rapid identification of strains were performed as described by Buchanan and Gibbon, [23]. The first step of biochemical characterization concerned the oxidation of both acetic and lactic acids which allow to distinguish strains belonging to *Acetobacter* genus from those belonging to *Gluconobacter* genus. The test of oxidation was performed in HS broth medium containing 0.05 % glucose and 1 % acetic acid or lactic acid as carbon source. Addition of acetic acid or lactic acid to the HS broth provokes the change of the green medium color into yellow [19]. One fresh colony of acetic acid bacteria was inoculated in 3 mL of HS broth and then incubated at 30 °C for 48 hours in aerobic atmosphere. Oxidation of acetic acid or lactic acid was assessed by the change of medium color back to green, due to pH rise.

Next, a panel of biochemical test performed, included sugars metabolism particularly galactose and mannose, acid production from glucose, nitrate reduction and ketogenesis from glycerol and mannitol.

The test of carbohydrate catabolism was carried out in basal liquid medium containing peptone (3 %); $K_2HPO_4$ (0.05 %); $KH_2PO_4$(0.05 %);$MgSO_4$ (0.01 %); $(NH_4)_2SO_4$(0.14 %) with 1 % of tested sugar as sole source of carbon. Acid production from glucose was tested in HS medium supplemented with 0.0016 % of bromocresol green and 1% of the sugar was used. The test of nitrate reduction was performed in liquid medium containing 0.3 % yeast extract, 0.5 % peptone and 0.1 % potassium nitrate. After 48 hours incubation at 30 °C, nitrate reduction into nitrite was revealed by addition of Griess reagent (Sigma-Aldrich,

Abidjan). Ketogenesis from glycerol and mannitol was detected by the methods outlined by Carr,[24].

#### *2.2.3. Analysis of Strains Resistance to Fermentative Stress*

***(i). Resistance to Alcoholic and Acid Stress***

Evaluation of resistance to ethanol, lactic acid, acetic acid and citric acid stress was carried out on YEP agar medium as described by Pereira et al. [25] and modified by Samagaci et al. [26].To prepare the stress medium, the YEP medium was cooled after autoclaving and maintained in liquid state at 45 °C in water bath and then appropriate quantity of alcohol or acid was aseptically added to the medium to obtain the fixed concentration. The concentration of ethanol, lactic acid, acetic acid and citric acid were added with different final concentrations (v/v) ranging from 0.1 to 20 % depending on the compound. The negative control was prepared in the same conditions except that it did not contain the corresponding compound studied. A standard initial load of bacterial inoculum was prepared by suspending a 16-24 h old colonies in 5 mL of sterile saline buffer to obtained an $OD_{600}$ = 0.1. Then this suspension was $10^6$-fold diluted and 100 µL of this diluted bacterial suspension was used as inoculum. The plate were then seeded with approximately the same bacterial load and incubated at 30 °C for 3 days in aerobic conditions. The capacity of strains to resist to alcoholic and acid stress is assessed by the growth of colony and the survival rate was determined after colonies count according to the following relation.

$$\text{Survival rate} = (\Sigma\text{AABcolony with alcohol or acid} / \Sigma\text{AABcolony in negative control}) \times 100)$$

***(ii). Effects of Temperature and pH on AAB Growth***

Effects of temperature and pH on the growth of AAB strains were analyzed in liquid medium as described by Pereira et al. [25] with slight modification. Bacterial cells were grown at 30 °C in YEPG medium until $OD_{600}$= 0.1, then 500 µL of this pre-culture were added in 10 mL of YEPG broth and then the cultures were incubated at different temperatures ranging from 30 to 50 °C.

To evaluate the effect of pH on AAB growth, the YEPG broth medium is adjusted to different pH (2; 3; 5; 7 and 8). The absorbance ($OD_{600}$) was measured at 6 h intervals to monitor the microbial growth during incubation.

***(iii). Influence of Sugar Concentration on AAB Growth***

The influence of sugar concentration on the growth of AAB was performed as described by Samagaci et al. [26] with modification. To prepare the culture medium, YEP broth was supplemented with glucose at different concentrations (5 %, 10 %, 15 %, 20 %, 25 % and 30 %). After sterilization, 9 mL of the medium is inoculated with 1 mL of AAB cells pre-culture ($OD_{600}$ = 0.5) and then incubated at 30 ºC for 7 days. The absorbance was measured at 600 nm against the sterile YEP medium to determine the turbidity, using a spectrophotometer PIOWAY Medical Lab - UV 752.

# 3. Results and Discussion

### *3.1. Screening of Producing AAB Strains*

From fermenting cocoa beans in three regions, a total of 444 AAB strains were isolated. This strains showed naturally acidification capacity but with different levels of acid production. Among these isolates, 97 strains showed the most important acidification capacity with clear halo diameter ranging from 1.7 to 3.1 cm. Moreover, 226 strains produced middle acidity with halo diameter in the range 1.1-1.7 cm and 85 isolates showed weak acidification capacity (Figure 1).

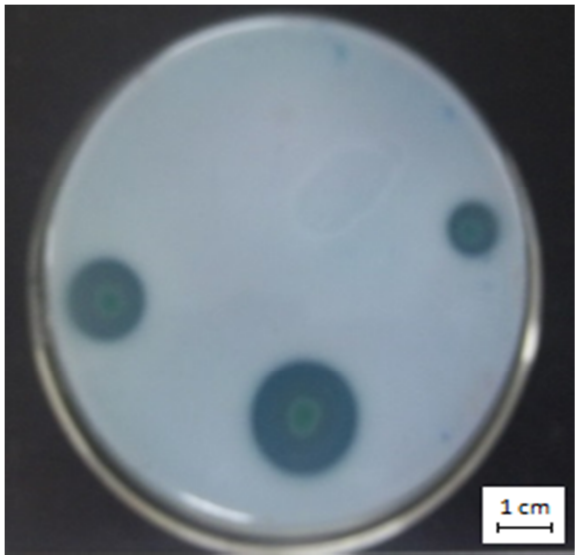


***Figure 1.*** *Screening of AAB with different levels of acid production.*

+ Strains were cultured on Hestrin-Schramm (HS) medium composed of 0.05 % glucose, 0.3 % peptone, 0.5 % yeast extract, 1.5 % $CaCO_3$, 1.2 % agar, and 4 % ethanol and incubated at 30°C for 10 days[21]. Acidifying capability of strains is characterized by a clear halo with a diameter related to the amount of acid produced.

The most acidifying strains (97) screened on solid medium, were further tested to evaluate the yield of acetic acid in liquid medium. Among these isolates, only 15 AAB strains yielded 2 to 38 g/L of acid (Table 1).The highest acid production (38.1 g/L) was achieved with the strain 121 D from Divo cocoa fermenting bean. Four (04) strains including 5 D; 56 AB; 139 D and 123 D also showed a good ability to produce acetic acid in liquid medium with 12.9, 13.4, 17.2 and 13.9 g /L of acid quantity respectively (Table 1). Acid production in these strains was characterized by a rapid decrease of the pH in the culture medium within 5 days dropping generally from 7.06 to 4.20 (data not shown).

The results show that although all the 444 strains analyzed have an acidification capacity, there is a difference in term of amount of acid produced. Hence the strains 121D as the most acidifying strain, yielded 38.1 g/L of acetic acid from ethanol. This production is particularly higher than that from AAB in Mexican cocoa fermentation reported by Romero-Cortes et al. [27]which was 25 g/L. However, Sharafi et al. [28] isolated AAB*(Acetobacterpasteurianus)* from fruit with

capacity to produce up to 100 g/L of acetic acid while Konaté et al. [29]reported AAB*(Acetobacterpasteurianus)* as the best acid producer from traditional palm wine producing 30 g/L of acetic acid. At date, the strain 121D isolated in this study, present the higher acetic acid production from cocoa fermentation.

The time course of acid production in these strains shows an irregular increase of acid amount in the liquid medium. A fair acid production characteristic of lag phase was observed in the four first days followed by a sharp rise of acid production in the period stretching from the fifth and the height day and become stable the remaining time at the maximum acid production (Figure 2). In solid medium, acid production was also assessed by an increase of halo diameter although a poor correlation was observed between the acid halo diameter in solid medium and the quantity of acid produced in liquid medium ($R^2$= 21.05).

***Table 1.*** *Acid production from different isolates on solid and in liquid medium.*

| Isolates | Clear halo diameter (en cm) | Amount of acid produced (g/L) |
|---|---|---|
| 168 AG (A.pasteurianius) | 3.10 ± 0.1 | 2.4 ± 0.0 |
| 154 AG (A.aceti) | 3.05 ± 0.1 | 2.1 ± 0.0 |
| 153 AG (A.pasteurianius) | 2.9 ± 0.1 | 3.2 ± 0.2 |
| 139 AG (A.aceti) | 2.85 ± 0.06 | 3.9 ± 0.0 |
| 140 AG (A.pasteurianius) | 2.55 ± 0.1 | 3.5 ± 0,2 |
| 52 AB (A.aceti) | 2.10 ± 0 | 5.7 ± 0.2 |
| 56 AB (A.peroxydans) | 2.10 ± 0.1 | 13.4± 0.2 |
| 139 D (A.aceti) | 2.05 ± 0.57 | 17.2± 0.2 |
| 22 AB (G.oxydans) | 1.90 ± 0.4 | 4.2 ± 0.3 |
| 123 D (A.pasteurianius) | 1.90 ± 0.57 | 13.9 ± 0.2 |
| 44 AB (Gluconobacter.sp) | 1.85 ± 0.1 | 5.0 ± 0,0 |
| 121 D (Acetobacter.sp) | 1.8 ± 0 | 38.1 ± 0.3 |
| 05 D (Gluconobacter.sp) | 1.8 ± 0.42 | 12.9 ± 0.3 |
| 08 AB (Gluconobacter.sp) | 1.75 ± 0.1 | 2.2 ± 0.2 |
| 49 D (G.oxydans) | 1.75 ± 0.21 | 8.2 ± 0.2 |

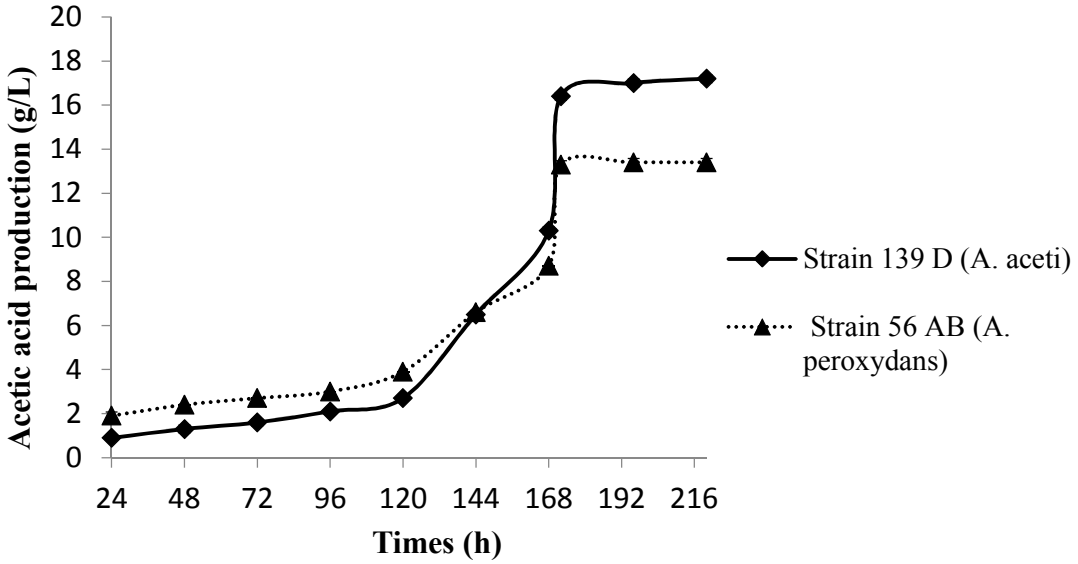


***Figure 2.*** *Time course of acid production during AAB growth.*

+ Strains were cultured in HS liquid medium at 30 °C under aerobic atmosphere by stirring at 130 rpm for 10 days. Acid yield was quantified in cell free supernatants by titration with NaOH (0.1 N) and phenolphthalein as a color indicator.

The lag phase for acid production presented by our AAB strains is relatively long comparatively to that observed in many strains. For instance, Lu et al.[30]reported an acid-producing *Acetobactersp* strains from spoiled banana in Taiwan, with short lag phase (12 h). Furthermore, no lag phase for acid production was observed in *Acetobacterpasteurianus* strains isolated from palm wine[29] and traditional rice fermentation[22].The long lag phase observed in our AAB strains studied, may be indicative of their behavior in their natural ecosystem (cocoa fermentation) and seems to be a common property for AAB involved in cocoa fermentation since Romero-Cortes et al.[27]also reported a long lag phase (2 days) in AAB strain *Acetobactertropicalis* from Mexican cocoa fermentation. In fact, maximum production of acetic acid during cocoa fermentation occurs generally after the third day [7, 25]and AAB strains should keep a low level of acid production until this period which corresponds to the occurrence of reactions leading to formation of cocoa and chocolate aroma precursors.

On the other hand, the sharp rise in acid production observed after the fifth day of AAB culture indicate a possible existence, of biological signal that surely accelerate the catabolism from ethanol leading to high acid yield. For instance, Lu et al. [30] reported that maximum acetic acid production is achieved during the stationary growth phase.

### *3.2. Biochemical Characterization of the Strains*

Acetic acid bacteria strains were identified as Gram

negative, short rod-shaped, catalase positive, oxidase negative and obligatory aerobic. These bacteria formed colonies surrounded by characteristic yellow area on isolation medium. The 15 strains presenting highest acidifying capacity were subjected to further biochemical identification. Among these strains, 10 were able to further oxidise acetic acid and lactic acid into $CO_2$ and $H_2O$ and subsequently classified as *Acetobacter* whereas 5 strains were not able to; these strains belong therefore to *Gluconobacter*genus. Three species including *A. pasteurianus*, *A. aceti* and *A. peroxydans* were identified among the strains highly producing acetic acid among the genera *Acetobacter* strains (Table 2).

All the *Acetobacter* strains presented the ability to grow at more than 10 % ethanol concentration. *Acetobacterperoxydans* was particularly characterized by acid production from glucose contrary to the others *Acetobacter* strains, while *Acetobacteraceti* was distinguished from *Acetobacterpasteurianus* from its capability to grow on mannose and to realize ketogenesis from glycerol. Additionally, unlike the other *Acetobacter* strains, *Acetobacteraceti* was not able to reduce nitrate into nitrite. However, the strain 121D yielding the most important acidity, belong to*Acetobacter*genus but was not further identified at species level by the biochemical methods used in this study. This strain 121D was therefore considered as *Acetobacter sp*. Likewise, among the five *Gluconobacter* strains only two isolates including 22 AB and 49 D were identified as *G. oxydans* the other strains were considered as *Gluconobactersp*(Table 2). The presence of *Acetobacterpasteurianus* and *Acetobacteraceti* among the best acid producers is consistent with previous studies reporting that, these species are generally known to be acid producer. Moreover, *Acetobacterpasteurianus* was assumed to be important for a successful cocoa bean fermentation process[31], probably due to its ability to produce acid as shown in this study. However, the results also show that the production of acetic acid in AAB strain from cocoa fermentation is not species-dependent since the yield of acid by strains belonging to the same species is extremely variable (Table 1).

Although the microflora characterized is limited, the results may be indicative of a certain diversity of AAB strains involved in Ivorian cocoa fermentation. These species were also found in Ghanaian, Brazilian and Mexican cocoa fermentation[11, 9,2].

***Table 2.*** *Biochemical properties of AAB strains isolated.*

| Isolates | Oxidation of lactic acid | Oxidation of acetic acid | Growth in 10 % ethanol | Growth on manose | Growth on galactose |
|---|---|---|---|---|---|
| 153 AG | + | + | + | + | |
| 154 AG | + | + | + | + | |
| 52 AB | + | + | - | + | |
| 140 AG | + | + | + | | |
| 56 AB | + | + | - | + | + |
| 139 AG | + | + | + | + | |
| 168 AG | + | + | + | + | |
| 121 D | + | + | - | _ | - |
| 123 D | + | + | + | + | |
| 139 D | + | + | - | + | |
| 49 D | - | - | - | + | + |
| 08 AB | - | - | - | + | + |
| 05 D | - | - | - | - | + |
| 22 AB | - | - | - | + | + |
| 44 AB | - | - | - | + | + |

***Table 2.*** *Continue.*

| Isolates | Nitrate reduction | Ketogenesis from mannitol | Ketogenesis from glycerol | Acid production from D-glucose | AAB species |
|---|---|---|---|---|---|
| 153 AG | + | - | - | + | *A.pasteurianius* |
| 154 AG | - | + | + | + | *A.aceti* |
| 52 AB | - | + | + | + | *A.aceti* |
| 140 AG | + | - | - | + | *A.pasteurianius* |
| 56 AB | + | + | - | - | *A.peroxydans* |
| 139 AG | - | + | + | + | *A.aceti* |
| 168 AG | + | - | - | + | *A.pasteurianius* |
| 121 D | + | ND | ND | + | |
| 123 D | + | - | - | + | *A.pasteurianius* |
| 139 D | - | + | + | + | *A.aceti* |
| 49 D | | + | + | | *G. oxydans* |
| 08 AB | | *ND* | *ND* | | *Gluconobacter sp* |
| 05 D | | *ND* | *ND* | | *Gluconobacter sp* |
| 22 AB | | + | + | | *G. oxydanssp* |
| 44 AB | | *ND* | *ND* | | *Gluconobacter sp* |

ND : not deterninate, + : positive, -: negative

### *3.3. Survival of AAB Strains Under Stress Conditions*

#### *3.3.1. Alcohol Tolerance of AAB Strains*

To study the response of AAB strains to environmental stress conditions, 4 isolates presenting an important acidification capacity notably 123 D (*A. pasteurianus*), 139 D (*A. aceti*), 56 AB (*A. peroxydans*) and 49 D (*G. oxydans*) were selected as models. The results show that all the 4 strains are able to grow on medium containing up to 8 % alcohol with a growth rate ranging from 11 to 66 % (Figure 3A). However, two trends were observed concerning the ability of strains studied to grow under strong alcoholic conditions. First, *Acetobacteraceti* strain 139 D displayed the capacity to grow in alcohol at concentrations up to 12 %. At this concentration, the strains retained 45 % of survival growth rate. However, beyond this concentration, a drastic decrease of survival capacity was observed. The second group including the strains 49 D (*Gluconobacteroxydans*), 123 D (*Acetobacterpasteurianus*), 56 AB *(Acetobacetrperoxydans)*, and 121 D *(Acetobactersp)* was characterized by a more sensitivity to alcoholic conditions. At 8 % of alcohol, these strains kept less than 30 % of survival growth rate. Moreover, the best acid producer *Acetobactersp* strain 121 D proved to be the less tolerant to alcohol. All the strains studied, failed to grow in medium containing more than 15 % of alcohol.

Our results are in agreement with those of Vaughn,[32]indicating that the maximum alcohol concentration tolerated by acetic bacteria is between 14 and 15%. These findings are also comparable to those of Sharafiet al.[28]who reported isolation of AAB strains from fruit with ability to grow at 11 % v/v of ethanol. Previously, Du Toit and Pretorius,[33] observed that AAB remained viable in wine with 14 % of ethanol.

Since alcohol concentration in the fermenting mass do not exceed 8 %, it is clear that the AAB strains isolated present an interesting behavior since they should be amply able to grow under alcoholic stress occurring in controlled cocoa fermentation. Ability to alcohol tolerance should be linked to their capacity to eliminate alcohol through oxidation into acetic acid. This propertie should be profitable since acetic acid formed is of great importance for quality of products.

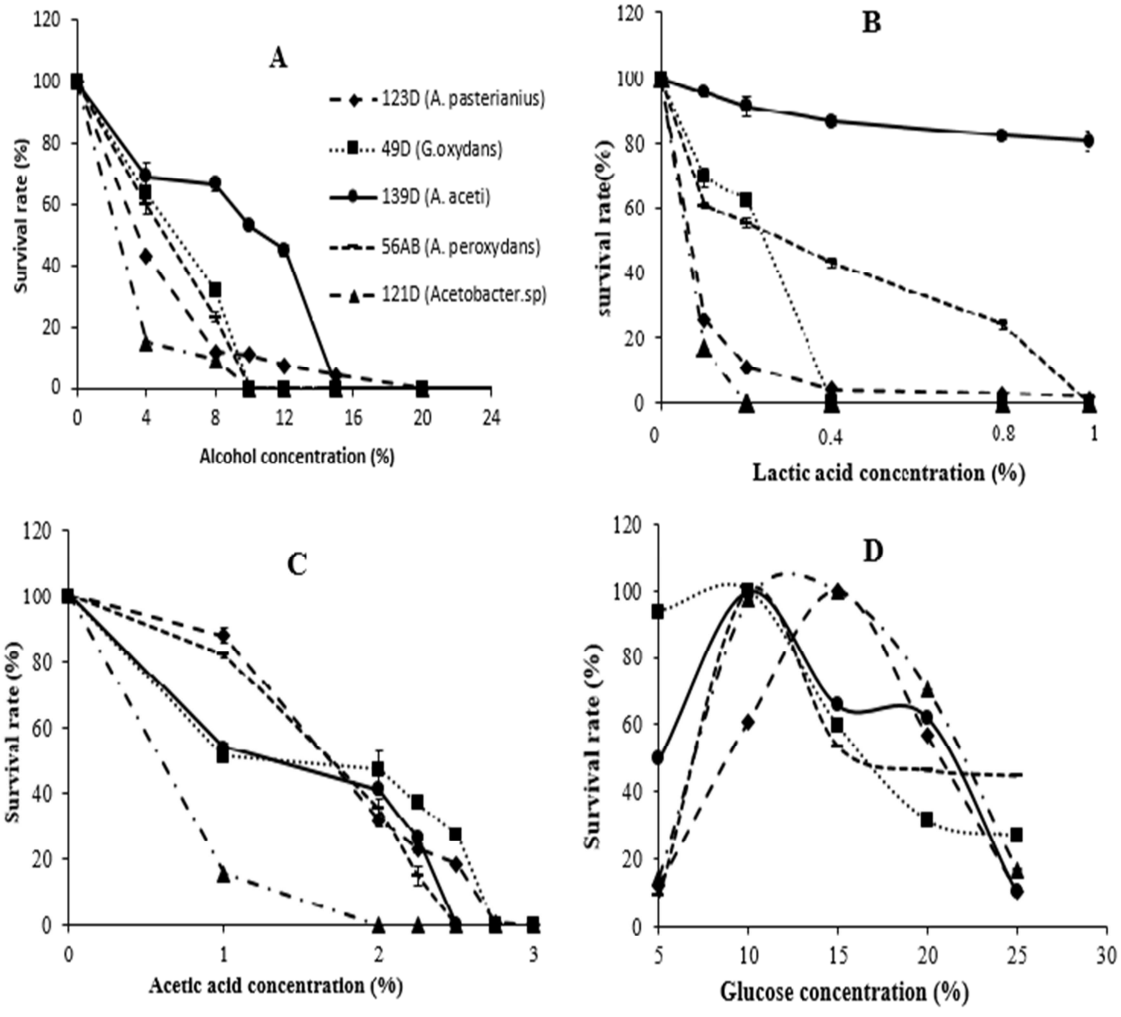


***Figure 3.*** *Effect of different compounds on AAB growth in vitro.*

+ Strains were grown on HS plate supplemented with corresponding volume of the compound to be tested and the plate was incubated at 30 ºC for 72 h. After incubation, microbial numeration was performed and the survival rate (%) was calculated using 0 % of compound as negative control except glucose for which the negative control was 15% corresponding to optimum bacterial growth. Error bars indicate standard deviations between three replicates.

### *3.3.2. Response of AAB to Acid Stress*

Two groups of strains were observed concerning their ability to grow under acid condition induced by lactic acid. The first group including only *Acetobacteraceti* strain 139 D was characterized by a strong tolerance to lactic acid, keeping more than 80 % of survival growth rate under 1 % lactic acid concentration (Figure 3B). This strain was able to conserve up to 30 % of survival growth rate at 2 % lactic acid (data not shown).

The second group was constituted by the strains 49 D (*Gluconobacteroxydans*), 123 D (*Acetobacterpasteurianus*), 56 AB (*Acetobacterperoxydans*), and 121 D (*Acetobactersp*). These strains were characterized by a poor tolerance to lactic acid comparatively to the first group, failing to grow at 1 %. This is materialized by a sharp decrease of bacterial growth rate from 100 to 40 % due to a small augmentation of lactic acid concentration from 0 to 0.4 %. The more acidifying *Acetobacteraceti* strain 121 D proved to be the most sensitive to lactic acid among the strains analyzed (Figure 3B).

The same grouping was observed when bacterial strains are grown in citric acid (data not shown). However, the strains analyzed revealed to be more tolerant to acetic acid than the other acids. Figure 3C shows that all the strains grow at more than 2 % of acetic acid apart from the *Acetobactersp* strain 121 D, the most acidifying strain. While the *Acetobactersp* strain 121 D failed to grow at 2 % of acetic acid, the other strains have a survival rate comprised between 14 and 47 %. No bacterial growth was observed at 3 % acetic acid.

Since, acidity from acetic acid in cocoa fermentation, is very important for obtaining a high quality of chocolate [2,14], it could be assumed that fermentation containing high acid-producing AAB strains such as 121 D, is more liable to generate high precursors content due to acidic activation of complexes enzymes into beans namely aspartic endoprotease and serine carboxypetidase. To this point of view, the screening of microbial strains with particular performance is of great importance. However, a starter should also be able to survive under environmental stress. Hence, although the strains 121 D yielded the most important amount of acid, this strain should not be a valuable starter due to its poor resistance to acid stress.

Moreover, lactic acid proved to have the most hindering effect among the acids tested on AAB growth. The general mechanism of adaptation to environmental stress is very complex and involve a sigma factor protein that is aided by alarmones of stringent response namely hyper-phosphorylated guanosine nucleotides[34]. The effectiveness of a bacterial response is therefore determined by the functioning of this system.

### *3.3.3. Effects of Temperature and pH on AAB Growth*

Table 3 shows that AAB strains 123 D and 49 D present maximal growth at 35 °C displaying a turbidity $OD_{600}$ of 2.34 and 1.23, respectively, although the growth was already at high level at 30 °C ($OD_{600}$ = 2.72). In contrast, the strains 139 D and 56 AB presented maximal growth at 30 °C ($OD_{600}$ = 1.65 and 1.99 respectively), but recorded a strong decrease of bacterial growth at 35 °C ($OD_{600}$ = 0.53 and 0.44 respectively). Furthermore, it was observed that most of strains analyzed present a growth at 45 and 50 °C as indicated by the turbidity comparatively to the negative control. However, the growth at these temperatures (45 and 50 °C) remained 2 to 20-folds lower than that observed at optimal temperatures. The lower survival rate for the temperatures beyond 40 °C suggests that our AAB strains may not be fully viable at these temperatures in fermentation conditions. In fact, the growth of acetic acid bacteria is associated with an exothermic process in the fermentation heaps which raises the temperature of fermenting beans up to 40 °C at 72 h [8]. The elevation of the temperature could be linked to the production of acid by AAB. Lu et al.[35] also found that thermotolerant*Acetobacter* strain produced more acetic acid (up to 41 g / L) in comparison with two non thermotolerant strains. So beyond 40 °C, a low population level may be responsible for a high specific production of acid.

***Table 3.*** *Effect oftemperature on AAB growth in vitro.*

| Strains | species | 30 $^0$c | 35 $^0$c | 40 $^0$c | 45 $^0$c | 50 $^0$c |
|---|---|---|---|---|---|---|
| 123 D | *A. pasteurianus* | 2.172± 0.15 | 2.544± 0.056 | 1.074± 0.074 | 0.00±0.00 | 0.00± 0.00 |
| 49 D | *G. oxydans* | 1.259±0.011 | 1.328±0.092 | 0.791±0.038 | 0.691±0.109 | 0.428±0.013 |
| 139 D | *A. aceti* | 1.655±0.036 | 0.537±0.063 | 0.405±0.003 | 0.358±0.003 | 0.114±0.006 |
| 56 AB | *A. peroxydans* | 1.992±0.013 | 0.445±0.002 | 0.377±0.070 | 0.187±0.014 | 0.132±0.009 |

+ The AAB strains were grown in YEPG broth and then the cultures were incubated at different temperatures during 72 h. The absorbance ($OD_{600}$) was measured at 6 h intervals to monitor the microbial growth during incubation.

Concerning the effect of pH, maximum growth was observed at pH 5 for all the strains analyzed except the strain 123 D which presented a higher growth rate at alkaline pH (8). Nearly, no growth was observed at pH 3 and below.

However, strains presenting maximum growth at acidic pH still have a relatively high growth at alkaline pH. Hence acidic pH have more severe effect on bacterial growth than basic pH (Table 4).

The growth of isolates at different pH is confirmed by Du Toit and Pretorius,[33]who observed an increase of AAB growth in wine, vinegar and beer at pH ranging from 2 to 6.5. It was also indicated that optimum pH for AAB growth is between 5.4 and 6.3[36]. However, the maximum growth rate occurring at pH 8, observed in *A. pasteurianus* isolated, constitute a particular trait. This could be explained by adaptation of this strain to cocoa fermentation conditions. Indeed, many studies reported the particular increase of pH to 7.9-8 at the end of spontaneous cocoa fermentation in Côte d'Ivoire[37,19,38].

***Table 4.*** *Effect of pH on AAB growth in vitro.*

| Strains | species | pH 2 | pH 3 | pH 5 | pH 7 | pH 8 |
|---|---|---|---|---|---|---|
| 123 D | *A. pasteurianus* | 0.050±0.001 | 0.050±0.006 | 1.964±0.117 | 2.176±0.005 | 2.562±0.014 |
| 49 D | *G. oxydans* | 0.076±0.032 | 0.149±0.121 | 2.196±0.008 | 1.915±0.018 | 0.744±0.007 |
| 139 D | *A. aceti* | 0.047±0.007 | 0.129±0.029 | 0.333±0.004 | 0.265±0.005 | 0.129±0.008 |
| 56 AB | *A. peroxydans* | 0.036±0.004 | 0.066±0.005 | 0.815±0.006 | 0.874±0.004 | 0.447±0.024 |

+ The AAB strains were grown YEPG broth adjusted to different pH was incubated at 30 ºC for 72 h. The absorbance ($OD_{600}$) was measured at 6 h intervals to monitor the microbial growth during incubation.

#### *3.3.4. Effect of Osmotic Stress Induced by Glucose on AAB Growth*

Maximum growth was obtained at 10 % of glucose concentration for all the strains excepted the strain 123 D *Acetobacterpasteurianus*. However, the growth is still relatively high at sugar concentrations in the range 10 to 15 %, with relative growth between 60 and 100 % (Figure 3D). Moreover, above 25 % of glucose concentration, bacterial growth is severely affected.

## 4. Conclusion

In this study, acetic acid bacteria presenting high acidification capacity involved in Ivorian cocoa fermentation have been characterized and their ability to grow under fermentative stress conditions have been evaluated. Acetic acid bacteria isolated from Ivorian cocoa fermentation present different levels of acid production, some yielding more acid than others. Different species namely *Acetobacteraceti,A. peroxydans*, *A. Pasteurianus* and *Gluconobacteroxydans* were found to be acid producers. The acidifying AAB strains behaved differently under different stress conditions. Lactic acid was found to have the most hindering effect on the growth of AAB.This study outline a certain diversity of AAB involved in Ivoirian cocoa fermentation and shows the relevance to screen among them the most valuable strain as potential starter for improvement of cocoa fermentation.

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