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Research article

Microbial synergy between *Pichia kudriazevii* YS201 and *Bacillus subtilis* BS38 improves pulp degradation and aroma production in cocoa pulp simulation medium

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ABSTRACT

Interactions between two major microorganisms from Ivorian cocoa fermentation, namely *Bacillus subtilis* BS38 and *Pichia kudriazevii* YS201, were investigated during fermentation in cocoa pulp simulation medium. The strains were mutually inhibitory, with *Bacillus* being more susceptible to this antagonistic effect than *Pichia*. However, both strains yielded different pulp-degrading enzymes, namely polygalacturonase (PG) from *Pichia* and pectate lyase (Pel) from *Bacillus*, that cooperate to efficiently breakdown pectin and vegetable pulp. The quantification of aromas from microbial cultures using Gas Chromatography-Mass Spectroscopy (GC-MS) coupled with headspace microextration (SPME) method, showed that *P. kudriazevii* produce mainly alcohols such as ethanol (63.165 g/L), phenylethanol (1.005 g/L), methylbutanol (0.138 g/L) and esters, notably ethyl acetate (0.037 g/L) and isoamyl acetate (0.032 g/L). The volatile fraction produced by *Bacillus* was dominated by butanediol (5.707 g/L), acetoin (1.933 g/L), phenylethanol (0.035 g/L) and acetic acid (0.034 g/L). In co-culture, *Bacillus* produced low levels of aroma compounds whereas a moderate decrease in the production of these compounds was observed in the yeasts strain. Thus, the dominant aromas present in the co-culture were mainly those from the yeasts strain; however, a 1.37 fold increase of ethanol production was observed in co-culture indicating a synergy between the strains. This study showed that cooperation between *B. subtilis* BS38 and *P. kudriazevii* YS201 leads principally to increasing pulp degradation and ethanol production, known as desirable properties for a well processing of cocoa fermentation.

1. Introduction

Microorganisms play a key role in the fermentation process of cocoa and are known to strongly impact the quality of finished chocolate (Meersman et al., 2016; Ouattara et al., 2008; Schwan and Wheals, 2004). These microorganisms generally belong to the following four groups: yeasts, acetic acid bacteria (AAB), lactic acid bacteria (LAB) and *Bacillus*. These microorganisms constitute the autochthonous microbiota responsible for natural cocoa fermentation (Ardhana and Fleet, 2003; Lefeber et al., 2010; Ouattara et al., 2011, 2017a,b; Pereira et al., 2012). During fermentation, sugars contained in the pulp are fermented into ethanol and lactic acid by yeasts and LAB, respectively, and AAB further transform ethanol into acetic acid (De Vuyst and Weckx, 2016; Ouattara et al., 2017a,b; Schwan, 1998; Soumahoro et al., 2015). Yeasts, together with *Bacillus*, produce enzymes that lead to pulp degradation (Cempaka et al., 2014; Ouattara et al., 2008; Schwan et al., 1995; Schwan and Wheals, 2004). This process is essential for the penetration of compounds into beans, principally ethanol and acetic acid. These compounds in turn activate pH-dependent proteolysis enzymes, leading to the degradation of the storage proteins into amino acids and peptides that are aroma precursors of chocolate (Schwan and Wheals, 2004; Voigt and Biehl, 1995; Voigt et al., 1994). A well-developed fermentation leads to a characteristic chocolate flavor and brown color of beans, as well as reducing their bitterness and astringency (Afoakwa et al., 2008, 2015; Aprotosoaie et al., 2016; Beckett, 2009).

Microorganisms are required for proper processing of cocoa into chocolate (Aprotosoaie et al., 2016; De Vuyst and Weckx, 2016; Schwan and Wheals, 2004), and the quality of the final product depends upon the metabolic traits of organisms selected for during fermentation (Crafack et al., 2013; De Vuyst and Weckx, 2016; Schwan and Wheals, 2004). In

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many parts of the world, the reliance on natural fermentation leads to a process that is difficult to control, resulting in variable quality of cocoa (Lagunes Gálvez et al., 2007). The use of defined starter strains is expected to provide better control over the fermentation process (De Vuyst and Weckx, 2016; Leal et al., 2008; Schwan and Wheals, 2004); however, to date there has been limited success in using defined starter strains to create a product that is superior to those produced by spontaneous fermentations (Lefeber et al., 2012; Crafack et al., 2013; Batista et al., 2016). Previous work often relied on the use of starter strains in pure culture, however here we propose that the limited success with defined starters in cocoa fermentation is due, in part, to a lack of understanding of how these cultures interact during cocoa fermentation. In support of this hypothesis, previous studies have reported that strain performance differs in single versus mixed culture (Qu et al., 2012; Rikhvanov et al., 1999; Van Rijswijck et al., 2017).

The objective of this paper was to analyze the nature of interactions between *Pichia kudriazevii* YS201 and *Bacillus subtilis* BS38 which were previously isolated from fermenting cocoa (Ouattara et al., 2011; Samagaci et al., 2016). We hypothesized that the growth rate, production of flavor precursors, and rate of pulp degradation would differ when these organisms were grown together, compared to as single cultures.

2. Material and methods

2.1. Microbial strains and culture medium

The yeast strain *P. kudriazevii* Ys201 and the bacterium *B. subtilis* BS38 were previously isolated from fermenting cocoa in Côte d'Ivoire (Ouattara et al., 2011; Samagaci et al., 2016). These strains were stored at -80 °C in 20 % glycerol.

All the microbial cultures were performed using cocoa pulp simulation medium (PSM) (Lefeber et al., 2010; Pettipher, 1986) with slight modifications. The PSM contained 3 % fructose; 2 % glucose; 1 % sucrose; 1 % pectin; 1 % citric acid; 0.6 % yeast extracts; 0.6 % soya peptone; 0.2 % potassium sulphate; 0.2 % magnesium sulphate, 0.04 % manganese sulphate and 0.1 % Tween 80, adjusted to pH 6.0. The concentration for each compound (Sigma Aldrich, Pennsylvania, USA) is indicated as w/v.

2.2. Microbial growth analysis

The growth of strains individually or in co-culture was analyzed in 50 mL PSM medium in a 250 Erlenmeyer flask. Strains for these experiments were first prepared by growing *B. subtilis* BS38 and *P. kudriazevii* YS201 in 10 mL nutrient broth (Difco, Detroit, USA) or YPD broth (1 % yeast extract, 1% peptone and 2 % dextrose) respectively, at 30 °C with shaking at 150 rpm. After 12 h incubation, the cultures were diluted 1:2 with PSM and allowed to grow for another 12 h. These precultures were used to seed 50 mL PSM medium at 1:100 (v/v) followed by incubation at 30 °C for 4 days with agitation at 150 rpm. At 3 h intervals, microbial growth was evaluated by reading the absorbance OD_{600} of the culture using spectrophotometer (Beckman Coulter, model DU-730). For accurate reading of absorbance, cultures were diluted when necessary to have an OD_{600} below 0.4, as the relationship between OD_{600} and cell density is linear below this value (Widdel, 2010). Additionally, microbial enumeration was performed by plate count at each growth phase, using the decimal dilution method (Samagaci et al., 2016). The viable cells count (expressed as colony forming units per milliliter; CFU/mL) of *P. kudriazevii* YS201 was determined after plating on YGC (Yeast Glucose Chloramphenicol), which inhibits *B. subtilis*, while nutrient agar (NA) supplemented to 100 µg/mL Nystatin (Fisher, USA) was used to selectively quantify *B. subtilis*.

Microbial growth rate was calculated during exponential phase; it was expressed as:

$\mu\ (h^{-1}) = [\log (N_2) - \log (N_1)]/ (T_2 - T_1)$ where N_1 is the number of CFU at time T_1 and N_2 the number of CFU at time T_2 (Dalgaard and Koutsoumanis, 2001). Cultures were duplicate in an experimental set up composed of 6 vials (two vials for each culture). Lag time was estimated by extrapolating the tangent at the exponential part of the growth curve, back to the inoculum level (Dalgaard and Koutsoumanis, 2001).

2.3. Protein purification

For enzymes purification, *B. subtilis* BS38 and *P. kudriazevii* YS201 strains were grown in 500 mL of PSM at 30 °C for 48 h. After centrifugation (5,000 × *g*) for 10 min, the supernatant was subjected to ammonium sulphate precipitation at 100 % saturation. The precipitated proteins were next dissolved in 10 mL distilled water and dialyzed overnight at 4–8 °C in a 14 kDa molecular weight cut off (MWCO) dialysis bag (Fisher, USA). The dialysate from *B. subtilis* BS38 supernatant was applied to fast flow CM Sepharose cation exchange resin (GE Healthcare, USA) previously equilibrated with 0.1 M Tris-HCl buffer, pH 7.2, whereas the dialysate from *P. kudriazevii* YS201 supernatant was applied to fast flow DEAE, Sepharose anion exchange resin (GE Healthcare, USA), equilibrated with 0.1 M citrate phosphate buffer pH 6.8. Chromatography was performed on 25 mL bed volume of resin contained in a gravity Econo column®, 30 cm length, 2.5 cm internal diameter (Bio-Rad, USA). The different protein fractions (10 mL) were eluted with increasing concentrations of NaCl from 0 to 500 mM. The purity of enzymatic proteins was assessed by electrophoresis using SDS and 12 % polyacrylamide gel. Molecular markers were used to approximately calculate the molecular weight of the proteins (Bio Rad Precision Plus Protein™, USA). Protein bands were detected by Coomassie blue staining, and the concentration was estimated using the Bradford reagent (Sigma Millipore, Bellefonte, USA).

2.4. Enzymatic assays

Pectinolytic enzymes were assayed in 1 mL of reaction mixture containing 0.2 % (w/v) apple pectin (Sigma Aldrich, Pennsylvania, USA), and 0.1M Tris-HCl pH 8 or citrate-phosphate pH 5. The reactions were run for 1 h, at 40 °C and enzymatic activities were measured spectrophometrically (OD_{540}) following the release of reducing group from pectin degradation using the dinitrosalicylic (DNS) acid method (Miller, 1959). One unit of polygalacturonase or pectate lyase was defined as the amount of enzyme that releases 1 µmol of galacturonic acid (GA) per minute, under experimental conditions.

2.5. Thin layer chromatography (TLC)

TLC was performed as described previously by Lojkowska et al. (1995). Pectin was digested by each purified enzyme at 0.05 µg/ mL, in a 100 µL reaction mixture as described above. Incubation was performed overnight at 45 °C. Next, 20 µL of the reaction products were applied on a silica gel chromatography sheet (10 cm Width, and 20 cm height) (Merk, Germany) and separated for 5 h using a mobile phase composed of n-butanol – acetic acid – water (5: 2: 3). The sheet was air dried at room temperature, and then immerged in a revelation solution containing phosphomolybdic acid - sulfuric acid – ethanol, (3: 10: 87). Finally, the sheet was plate heated at 100 °C to reveal the spot of the reaction products from pectin degradation.

2.6. Pulp degradation and maceration capacity of enzymes

The ability of microbial pectinolytic enzymes to macerate vegetable was analyzed using the epicarp and mesocarp of cucumber *(Cucumis sativus)* as solid substrate. Cucumber is a standard model generally used for studying native pulp degradation and maceration capacity of pectinolytic enzymes from microbial culture (Schwan et al., 1997; Khan and Latif, 2016). Each sample (of cucumber) weighting approximately 5 g was submerged in 10 mL of crude enzyme from the 48 h culture supernatant described above. The submerged sample was incubated for 24 h at

30 °C under shaking at 150 rpm. The negative control consisted of cucumber submerged in PSM (enzyme free). After incubation, the crude enzyme or the PSM was removed, replaced with 10 mL distilled water, and then vortexed at highest speed (VWR, Analog Vortex Mixer) for 1 min. The debris obtained from the macerated cucumber was centrifuged at 100 × *g* for 5 min.

Pectin degradation was also measured after incorporation of 0.2 % (w/v) apple pectin into 20 % agar, at pH 5.0 (citrate-phosphate buffer) or pH 8.0 (Tris-HCl, buffer). After solidification of the agar, 5 U of enzyme was spotted on pectinized plate agar, incubated 5 h at 30 °C. Then, the solid plate was flooded with lugol solution (Sigma Aldrich, Pennsylvania) to detect clearance zones corresponding to enzymatic activity (Ouattara et al., 2008).

2.7. Volatiles analysis

Volatile compounds produced by the strains were analyzed under single and co-culture conditions. *B. subtilis* BS38 and *P. kudriazevii* YS201 were grown at 30 °C for 18–24 h on NA and YPD agar, respectively. Colonies were picked and resuspended in sterile distilled water to an $OD_{600} = 0.05$. This suspension was used to inoculate to 1% (v/v), 5 mL of PSM medium contained within a 20 mL sterilized vial suitable for GC-MS analysis (Restek, USA). The vial was sealed with an 18 mm cap with a thermo-resistant silicon septum, and was incubated at 30 °C for 48 h with constant agitation at 150 rpm in orbital shaking incubator. The negative control was non-inoculated medium incubated under the same conditions. Cultures were prepared in triplicate.

The extraction of the volatile compounds was performed using solid phase microextraction of the vial headspace (SPME-HS) using a 50/30 μm divinylbenzene/ carboxene/ polydimethylsiloxane (DVB/CAR/ PDMS) fiber (Supelco,/Sigma Aldrich, Bellefonte, PA, USA) as previously described by Koné et al. (2015). The fiber was previously conditioned in the chromatograph injector at 250 °C for 3 min and then was exposed to the sample headspace at 50 °C for 45 min. Extracted volatile compounds were analyzed using an Agilent Technologies 7890A gas chromatograph (Agilent Technologies, Inc., Santa Clara, CA) equipped with a Gerstel MPS2 multipurpose autosampler (Gerstel GmbH & Co. KG. Mülheim an der Ruhr, Germany) that was coupled to an Agilent 5975C mass selective detector that was a Hewlett Packard DBWAX capillary column (30 m length × 0.25 mm internal diameter × 0.25 μm film thickness) (Agilent, Palo Alto, CA, USA). Agilent MassHunter GC/MS Acquisition software (Version B.07.00 SP2.1654) and Gerstel Maestro 1 software (Version 1.3.20.41) were used to control the instrument and autosampler, respectively.

The GC oven temperature was initially set at 40 °C, was held for 5 min, and then increased to 140 °C at a rate of 2 °C/min. Helium was used as the carrier gas at a flow rate of 1 mL min−1. Headspace samples were injected in splitless mode over the course of 2 min and the injector temperature was held at 250 °C.

Volatile compounds were putatively identified by probability based matching of their mass spectra with those obtained from a commercial database (Wiley275.L, HP product) and by matching the Kovac index (KI) of the compounds with literature values reported for equivalent columns. Whenever possible, the identification was confirmed by using pure reference standards of the components.

Relative quantification was performed from peak areas integration of well-identified compounds using the MSD Chemstation software (version E.02.02.1431, Agilent Technologies). Absolute quantification was performed using external and internal standards. External standard curves were obtained with good linearity over the relevant concentration range of the sample volatiles (R^2 0.997–0.999). Internal standards consisting of isotopically labeled forms of the compounds were used at constant concentrations to normalize the differences in the response of the GC detector in the vials (see supplemental material Table S1). Volatile concentrations were calculated based on linear regression equations derived from their standard curves.

Table 1. Microbial growth during fermentation in the different cultures.

Growth phases		Single culture *B. subtilis*		Single culture *P. kudriazevii*		Coculture			Theoretical expected growth in co-culture
		OD_{600}	Log (CFU/mL)	OD_{600}	Log (CFU/mL)	OD_{600}	*B. subtilis* Log (CFU/mL)	*P. kudriazevii* Log (CFU/mL)	OD_{600}
Lag	Start	**0.017** (±0.004)	**5.02** (±0.045)	**0.065** (±0.011)	**5.38** (±0.15)	**0.089** (±0.006)	**5.02** (±0.045)	**5.38** (±0.15)	**0.082** (±0.015)
	End	**0.05** (±0.008)	**5.62** (±0.08)	**0.158** (±0.019)	**5.84** (±0.115)	**0.212** (±0.019)	**5.60** (±0.071)	**5.79** (±0.095)	**0.208** (±0.027)
Exponential	End	**1.85** (±0.122)	**7.24** (±0.075)	**8.16** (±0.225)	**7.60** (±0.055)	**8.64** (±0.201)	**7.10** (±0.105)	**7.52** (±0.091)	**10.01** (±0.347)
Stationary	Late	**3.16** (±0.138)	**7.47** (±0.065)	**10.16** (±0.45)	**7.69** (±0.085)	**10.85** (±0.960)	**7.21** (±0.053)	**7.59** (±0.085)	**13.32** (±0.588)
Growth rate μ_{max} (h^{-1})		**0.373[a]** (±0.025)			**0.405[b]** (±0.013)		**0.345[c]** (±0.008)	**0.398[d]** (±0.011)	

Duration (start lag phase – end lag phase) = 2 h; (end lag phase – end exponential phase) = 10 h; (end exponential phase – late stationary phase) = 10 h; μ_{max} maximum growth rate measured during exponential phase. In the same row, numbers with different letters are significantly different. Cultures were duplicate in an experimental set up composed of 6 vials (two vials for each culture).

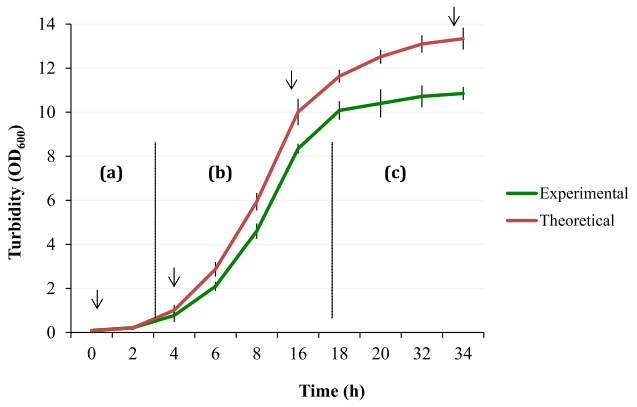

Figure 1. Comparative evolution of microbial growth in singles and co-culture. Red line is the sum of OD_{600} from the different single cultures as theoretical growth expected in co-culture. Blue line is the actual and experimental microbial growth measured in co-culture. Arrows indicate the time at which viable cells enumeration on plate agar was performed (see Table 1). (a) lag phase, (b) exponential phase, (c) stationary phase.

2.8. Statistical analysis

When required, a one-way analysis of variance (ANOVA) using StatPlus software version 5.4 at 95 % confidence was performed to determine whether there are any significant differences between the means of independent group of replicates.

3. Results

3.1. Co-culture negatively impacts the growth of both *B. subtilis* and *P. kudriazevii*

To analyze the interaction between *B. subtilis* and *P. kudriazevii* during co-culture, growth was compared between single- and co-culture. The initial OD_{600} for the single cultures was 0.017 for *B. subtilis* BS38 and 0.065 for *P. kudriazevii* YS201 and the OD_{600} from co-culture was 0.089, corresponding approximately to the sum of absorbances from the single cultures (Table 1). During the lag phase, the sum of OD_{600} from single cultures corresponded to the OD_{600} obtained in co-culture whereas differences increased between these two values during the rest of growth cycle suggesting decreased microbial growth in co-culture (Figure 1).

We further quantified the growth of each culture by performing viable cell counts on selective agar. We observed that the growth of *B. subtilis* BS38 in co-culture (μ_{max} of 0.345 ± 0.008 h^{-1}) was slower than growth in single culture (μ_{max} of 0.373 ± 0.025 h^{-1}) (Table 1). The viable cell counts of *B. subtilis* BS38 at the end of the exponential growth phase in co-culture was 7.1 ± 0.105 log CFU/mL thus representing 72 % of the bacterial load obtained in single culture, which was 7.2 ± 0.075 log CFU/mL. Likewise, the growth rate of *P. kudriazevii* YS201 decreased from 0.405 (±0.013) h^{-1} in single culture to 0.398 (±0.011) h^{-1} in co-culture (Table 1). The growth of this strain in co-culture reached 7.52 ± 0.091 log CFU/mL thus representing 83 % of the load obtained in single culture (7.6 ± 0.055 log CFU/mL). For both strains, the growth rates were significantly different in the two conditions (single and co-culture) as assessed by ANOVA.

3.2. Microbial growth analysis at different pH

Analysis of microbial growth in acidic range showed that at pH 4, *B. subtilis* reach only 4.82 % of the maximum OD_{600} obtained at pH 6 (Figure 2A). This poor growth of *Bacillus* was characterized by a long lag phase of 10 h and maximum OD_{600} of 0.168 (±0.021) (Figure S1). When, these *Bacillus* cells were transferred and grown at pH 6, they recovered their growth capacity. On the other hand, *P. kudriazevii* YS201 at pH 4, grew to a final OD_{600} that was approximately 60 % of that of cells grown at pH 6 (Figure 2A).

Given these results, two co-culture conditions were analyzed: co-culture at pH 4 where *Bacillus* and *Pichia* growth differed dramatically, and co-culture at pH 6 where both strains grew well. At pH 4, *P. kudriazevii* co-cultured with *B. subtilis* reached 7.42 ± 0.105 log CFU/mL after 24 h, thus representing 94.79 (±3.33) % of the density observed in single culture (Figure 2B), while the load of *Bacillus* in this co-culture was 6.07 ± 0.055 log CFU/ mL.

At pH 6, the growth of *P. kudriazevii* in the co-culture achieved a load of 7.55 ± 0.085 log CFU/ mL thus representing 78.15 (±4.78) % (Figure 2B) of cell concentration reached in single culture. *Bacillus* reached 7.17 ± 0.06 log CFU/ mL in this co-culture.

On the other hand, whatever the initial pH of the medium, it did not significantly vary at the end of the cultures. The more notable shift of pH was 6.07 (from initial pH 6.0), observed in *Bacillus* single culture and co-cultures.

3.3. Basic characteristic of purified pectinolytic enzymes

Enzymes from both strains were first purified to electrophoretic homogeneity. During the purification process, pectin degrading activity from *B. subtilis* BS38 weakly bound on CM sepharose, and eluted with a weak ionic strength of salt solution 0.1 M NaCl while a higher concentration of NaCl (0.45 M) was required to elute the enzyme produced by *P. kudriazevii* YS201 from DEAE sepharose. The enzyme from *B. subtilis* BS38 has a molecular weight of approximately 25 kDa, and split pectin to give products that strongly absorbed in the UV range (230 nm) (Figure S2). Addition of 0.1 mM calcium to the reaction mixture strongly

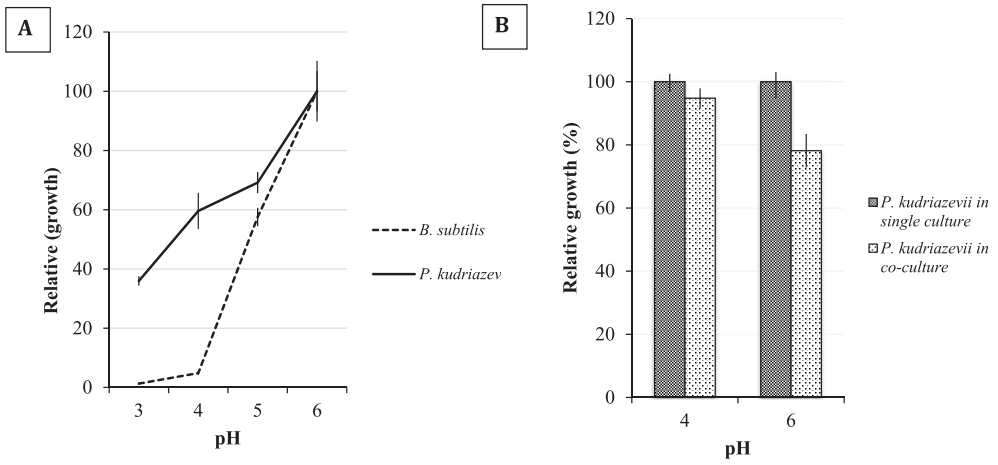

Figure 2. Growth of strains analyzed at different pH. A. Effect of pH on the growth of strains. OD_{600} obtained at the end of the cultures after 24 h, 30 °C, in PSM are shown. The relative growth was expressed as the percentage of absorbance using maximum OD_{600} at pH 6 as 100 %. B. Level of *P. kudriazevii* growth reached in single and co-culture at different pH. The relative growth was calculated as the percentage of viable cells (VC) obtained in co-culture using the load of VC in single culture as 100 %.

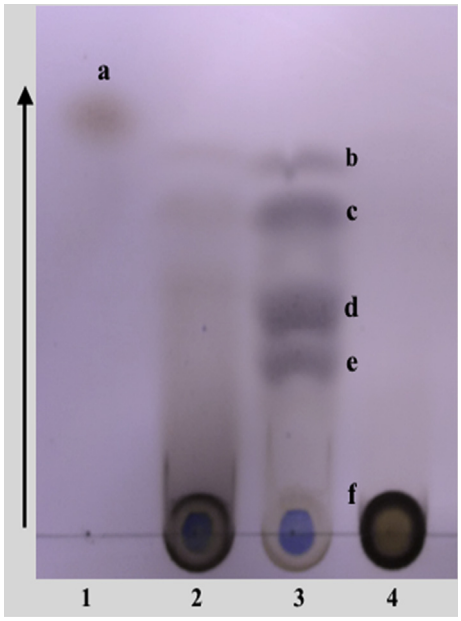

Figure 3. Thin layer chromatography showing the degradation products from pectin due to enzymes activity. a, b, c, d and e are different products from pectin degradation; **f** is the polymeric pectin that remained unmoved from the initial line during chromatography. Arrow indicates the migration direction. Pectin was incubated with 1.5 U of enzyme and incubated at 45 °C overnight. **Lane 1** monomeric galacturonic acid used as marker; lane 2, PG in 0.1 M acetate reaction buffer pH 5; lane 3 Pel in 0.1 M Tris-HCl reaction buffer pH 8; lane 4, mixed PG and PL in 0.1 M acetate reaction buffer pH 6. The non-degraded pectin (spot f) (lane 4) remains abundant comparatively to the pectin (lane 3) effectively degraded.

increases the activity of this enzyme showing calcium-dependence. Moreover, this enzyme was completely inhibited by 0.1 mM EDTA (Table S2). The enzyme from *B. subtilis* was therefore identified as a pectate lyase (Pel) (EC 3.2.1.15) that showed maximum activity at pH 8 in liquid phase reaction, with a large range of activity (pH 6.5–9) but total loss of activity below pH 6 (Figure S3). In contrast, pectinolytic enzyme from *P. kudriazevii* YS201 was identified as polygalacturonase (PG) (EC 4.2.2.2). This enzyme yields products that did not absorb light in the UV range or be inhibited by EDTA (Table S2). The purified PG had a molecular weight of approximately 31 Kda, a pH range of activity between 3 and 5.5 (Figure S3). No other pectinolytic enzyme such as pectin methyl esterase was found in the supernatants.

Results from thin layer chromatography showed that, PG and Pel individually at their respective optimum pH (5 and 8) degrade pectin; monomer was not detectable after digestion regarding the spot **a,** that was not present in the lane 2 and 3 (Figure 3). At pH 8, it was observed that Pel led to a sharp degradation of pectin represented by the spot **f,** which intensity was visibly weak (Figure 3, lane 3). In comparison, more pectin remained after PG digestion (pH 5) regarding the visible intensity of spot **f** (Figure 3, lane 2). Moreover, the breakdown of the pectin led to accumulation of degradation products that were more important with Pel action as showed the visible intensity of the spots **b, c, d** and **e,** lane 3, comparatively to PG action, lane 2 (Figure 3). When PG and Pel were mixed and incubated with pectic substrate in the same buffer at pH 6, no pectin degradation products were observed as the spot **b, c, d,** and **e** were not present in lane 4 (Figure 3). Indeed, the combined enzymes failed to degrade pectin at pH 6 in liquid phase.

3.4. Cooperative action of P. kudriazevii YS201 and B. subtilis BS38 leads to strong degradation of pectin

Due to their different characteristics at different pH (Figure S2), it was not possible to find a common active pH at which the combined action of these

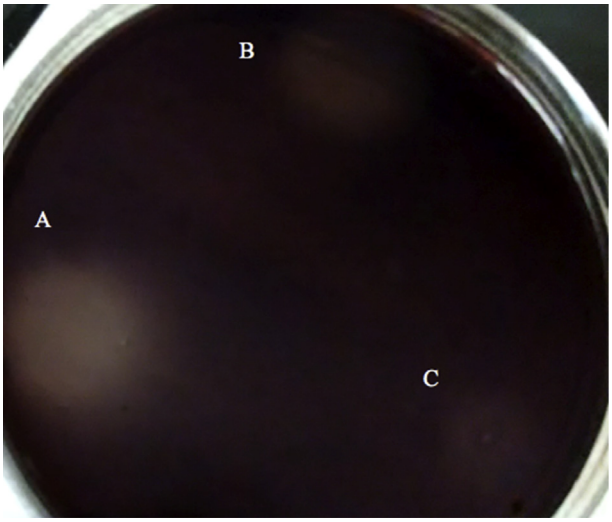

Figure 4. Cooperative degradation of pectin by different purified enzymes on solid phase. 5 U of enzymes were dropped on the dried solid phase containing 2 % pectin, 0.1 M acetate buffer pH 6, and incubation for 5 h at 30 °C. A: 2.5 U of each enzyme solution were mixed (Pel from *B. subtilis* + PG from *P. kudriazevii*) B: 5 U of Pel C: 5 U of PG.

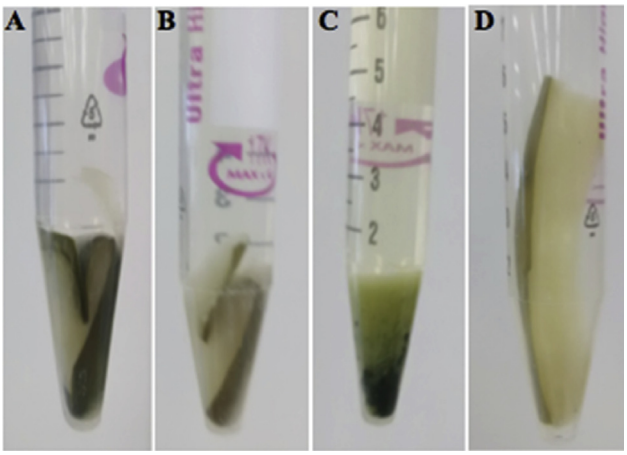

Figure 5. Maceration capacity of different crudes enzymes. A: crude enzyme from *B. subtlis*, B: crude enzyme from *P. kudriazevii* D: crude enzyme from co-culture diluted ½ with distilled water. C: control.

Table 2. Endogenous volatile compounds present in the medium.

Volatile in the PSM medium
2-methyl propanal butanal
2-methyl butanal
3-methyl butanal
n-hexanal
furaldehyde
nonanal
benzaldehyde

Non-inoculated medium contained in 20 mL sterilized vial (Restek, USA) tightly closed was incubated at 30 °C for 48 h, under shaking at 150 rpm. Volatiles were detected using GC-MS and identified by probability based matching of their mass spectra with those obtained from a commercial database (Wiley275.L, HP product) and by matching the Kovac index (KI) of the compounds with literature values reported for equivalent columns.

Table 3. Volatile compounds produced by *P. kudriazevii* strain studied.

	Volatile compound detected	Relative abundance (%)	Odor description	References
Alcohol	Ethanol	23.055 (±2.547)	–	–
	1-Butanol, 3 methyl	13.284 (±3.893)	Bitter, chocolate	Rodriguez-Campos et al., 2012
	1-Butanol, 2 methyl	6.068 (±0.956)	Fruity	Ramos et al., 2014
	Phenyl alcohol	28.961 (±8.212)	Floral	Rodriguez-Campos et al., 2012
Acids	Isovaleric acid*	–	Sweat, rancid	Rodriguez-Campos et al., 2012
	Acetic acid*	–	Sour, vinegar	Rodriguez-Campos et al., 2012
	2 Methyl butanoic acid*	–	Sweaty	Krings et al., 2006
Esters	Methyl acetate	–	–	–
	Ethyl acetate	22.048 (±2.529)	Fruity pineapple	Rodriguez-Campos et al., 2012
	Ethyl propanoate	0.962 (±0.422)	–	
	Isoamyl acetate	4.383 (±1.505)	Fruity, banana	Ramos et al., 2014
	Phenyl ethyl acetate	1.235 (±0.285)	Fruity sweet	Rodriguez-Campos et al., 2012
	Isobutyl acetate*	–	Fruity	Rodriguez-Campos et al., 2012
	Ethyl butanoate*	–	–	–
Others	Methionol 3 methyl thiol propanol*	–	–	–

Culture contained in 20 mL sterilized vial (Restek, USA) tightly closed was incubated at 30 °C for 48 h, under shaking at 150 rpm. For GC-MS analysis, the relative abundance of volatile compounds in each culture was calculated using area integration from chromatogram. Areas were normalized by using total areas from all volatiles compounds detected in the culture as 100 %. Standard deviations were obtained from three replications. The compounds constantly detected in all the cultures were considered for abundance calculation. (*) Compounds not constantly detected in the cultures. ± Standard deviation between three biological replications.

enzymes could be analyzed in liquid phase. We next tested activity on a solid support consisting of a buffered solution of pectin solidified with agar. The results show that Pel from *B. subtilis* BS38 was capable of degrading pectin on solid phase even at pH 5 (data not shown). Similarly, PG from *P. kudriazevii* YS201 was still active at pH 7.5 on solid phase. Therefore, the combined action of these enzymes was analyzed on pectin concentrated solid phase (2 %) at pH 6. The results showed that a mixture of both enzymes (Pel + PG) lead to a sharp degradation of pectin as showed the visible clear halos, comparatively to individual enzymes (Figure 4).

We further analyzed this cooperative effect by testing the supernatant directly from co-culture, using cucumber as a substrate. A complete disintegration of the vegetal tissue was achieved after 48 h contact with the co-culture supernatant whereas only a softening was obtained with the single culture supernatants (Figure 5).

3.5. General profile of aroma compounds yielded by *B. subtilis* BS38 and *P. kudriazevii* YS201

Analysis of volatiles produced by the strains in different cultures was carried out in two steps. In the first step, the compounds were identified based on the retention time and in the next step, we performed an absolute quantification of the analyts using standard curves. To avoid misinterpretation, volatile compounds detected in non-inoculated medium (negative control) were discarded from data analysis (Table 2).

The results indicated that the observed volatile compounds generated by *P. kudriazevii* YS201 were mainly alcohols and esters while acids were not detected (Table 3). Alcohols were mainly ethanol, methyl butanol and phenylethanol while ethyl acetate, isoamyl acetate and phenyl acetate composed the class of ester produced by this yeast strain (Table 3).

Table 4. Volatile compounds produced by *B. subtilis* strain studied.

	Volatile compounds detected	Relative abundance (%)	Odors description	References
Alcohols	1-Butanol	1.401 (±0.075)	–	–
	1-Butanol, 3 methyl	6.008 (±0.823)	Malty, chocolate	Rodriguez-Campos et al., 2012
	1-Butanol, 2 methyl	2.579 (±0.489)	Fruity	Ramos et al., 2014
	2, 3-Butanediol	47.458 (±10.260)	Sweet, chocolate	Ramos et al., 2014
	Ethyl hexanol*	–	Fruity, green	Bonvehí, 2005
	Phenol*	–	Smoky	Rodriguez-Campos et al., 2012
	Phenyl alcohol	1.755 (±0.476)	Floral	Rodriguez-Campos et al., 2012
Ketones	2,3 Butadione	5.770 (±1.019)	Buttery	Rodriguez-Campos et al., 2012
	3-Hydroxy, 2 butanone	28.432 (±7.572)	Buttery, creamy	Rodriguez-Campos et al., 2012
Acids	Acetic acid	5.267 (±1.837)	Sour, vinegar	Rodriguez-Campos et al., 2012
Esters	Isoamyl acetate*	–	Fruity, banana	Ramos et al., 2014
Pyrazines	Dimethyl ethyl pyrazine	1.325 (±0.115)		
Others	Methionol 3 methyl thiol propanol	–	–	–
	Furan methanol	–	Cooked sugar	Bonvehí, 2005
	Furan, 2 phenyl	–	–	–
	Furan, 2,5 dimethyl	–	–	–
	Propan, 2 chloro, 2 nitro	–	–	–
	Dimethyl disulfide	–	Sulfurous	Bonvehí, 2005

Culture contained in 20 mL sterilized vial (Restek, USA) tightly closed was incubated at 30 °C for 48 h, under shaking at 150 rpm. For GC-MS analysis, the relative abundance of volatile compounds in each culture was calculated using area integration from chromatogram. Areas were normalized by using total areas from all volatiles compounds detected in the culture as 100 %. Standard deviations were obtained from three replications. The compounds constantly detected in all the cultures were considered for abundance calculation. (*) Compounds not constantly detected in the cultures. ± Standard deviation between three biological replications.

Table 5. Absolute quantification of the main aroma produced by strains in single and co-culture.

Compounds	Aroma production (g/L)			Odor threshold (g/L)
	B. subtilis	*P. kudriazevii*	*B. subtilis* + *P. kudriazevii*	
Ethyl acetate	nd	0.037 (±0.002)	0.042 (±0.008)	0.006 National Institute of Health, 2015
Isoamyl acetate	0.004 (±0.000)[a]	0.032 (±0.008)[b]	0.020 (±0.003)[c]	34×10^{-5} National Institute of Health, 2015
Ethanol	nd	63.165 (±6.536)	86.596 (±4.177)	0.049 National Institute of Health, 2015
Butanediol	5.707 (±0.098)	nd	1.102 (±0.200)	–
Phenyl ethanol	0.035 (±0.003)[a]	1.005 (±0.082)[b]	0.482 (±0.022)[c]	0.007 National Institute of Health, 2015
2 methyl butanol	nd	0.042 (±0.001)	0.032 (±0.004)	12×10^{-5} National Institute of Health, 2015
3 methyl butanol	0.005 (± 0.000)[a]	0.138 (±0.042)[b]	0.116 (±0.017)[c]	0.3×10^{-5} Bonvehí, 2005
3 hydroxy 2 butanone	1.933 (±0.221)	nd	nd	–
Acetic acid	0.034 (±0.004)	nd	nd	0.032 Bonvehí, 2005

nd, not detected. Absolute quantification was performed using external and internal standards. Different concentrations of external standards allowed plotting standard curves. Internal standards were composed of isotope labeled form of the compounds that were used at constant concentrations to normalize the differences in the response of the GC detector in the vials. Then the concentrations were calculated from the linear regression equation of the standard curves. ± Standards deviation between three biological replications. In the same row, numbers with different letters are significantly different. NIH, National Institute of Health, (USA).

With respect to *B. subtilis* BS38, this strain produced a wider spectrum of volatile compounds that belong to the class of alcohols, ketones, acids, pyrazines and furans (Table 4). Alcohols presented a large proportion of these volatiles compound from *B. subtilis* BS38 with the yield of phenyl ethanol, 1-butanol, 3-methyl butanol, 2-methyl butanol and 2,3-butanediol. On the other hand, 2,3-butanedione and 3-hydroxy 2-butanone represented the class of ketones produced whereas acetic acid and dimethyl ethyl pyrazine composed the other class of volatile compound produced from this *Bacillus* strain (Table 4).

Some compounds were produced only by one strain. For instance, ethanol and all esters produced by *P. kudriazevii* YS201 were not observed in *B. subtilis* BS38 culture medium. Likewise, compounds such as butanol, butanediol, 2, 3-butanedione as well as 3-hydroxy 2 butadione and dimethyl ethyl pyrazine produced by *B. subtilis* BS38 were not be detected in *P. kudriazevii* YS201 cultures. On the other hand, the results showed that some compounds (e.g., phenyl alcohol, methylated butanol) were produced by both microbial strains. In co-culture the abundance of volatile compounds was different from that observed in single culture.

3.6. Interactions of strains in co-culture lead to the modification of aroma compounds production

Quantification analysis of the volatiles revealed that the studied microbial strains yielded an array of aroma compounds at concentrations above the known odor threshold (Table 5). In single culture, *P. kudriazevii* YS201 was able to produce up to 63.165 (±6.536) g/L ethanol. This production increased significantly in co-culture where it reached 86.596 (±4.177) g/L (Table 5). However, for the other volatiles, a general decrease was observed in co-culture. For instance, the production of phenylethanol and 3-methylbutanol, two of the main volatiles present in *P. kudriazevii* YS201 single culture, were observed at concentrations of 1.005 (±0.082) and 0.138 (±0.042) g/L, respectively. These levels in co-culture decreased to 0.482 (±0.022) and 0.116 (±0.017) g/L, respectively (Table 5 and Figure 6). Moreover, the decrease of aroma compounds production comparing single to co-culture was most dramatic with *B. subtilis* BS38, which produced butanediol (5.707 ± 0.098 g/L), 3-hydroxy 2-butanone (acetoin) (1.933 ± 0.221 g/L) and acetic acid (0.034 ± 0.004 g/L) in single culture but almost failed to produce these compounds in co-culture.

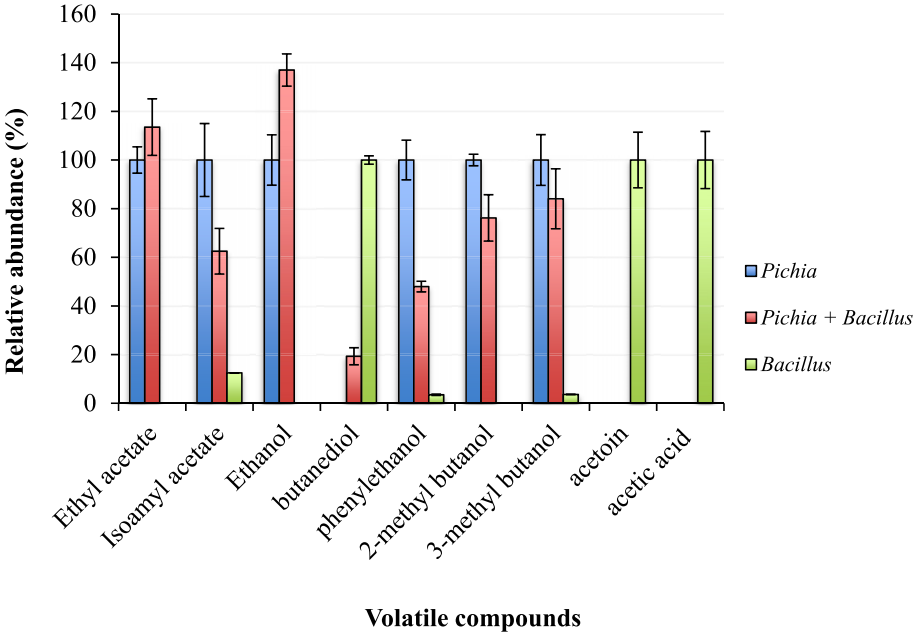

Figure 6. Comparative levels of aroma production in single and co-culture. Relative production was expressed as percentage, using the quantity of aroma produced in single culture as 100 %. Error bars indicate standard deviation between three replicates.

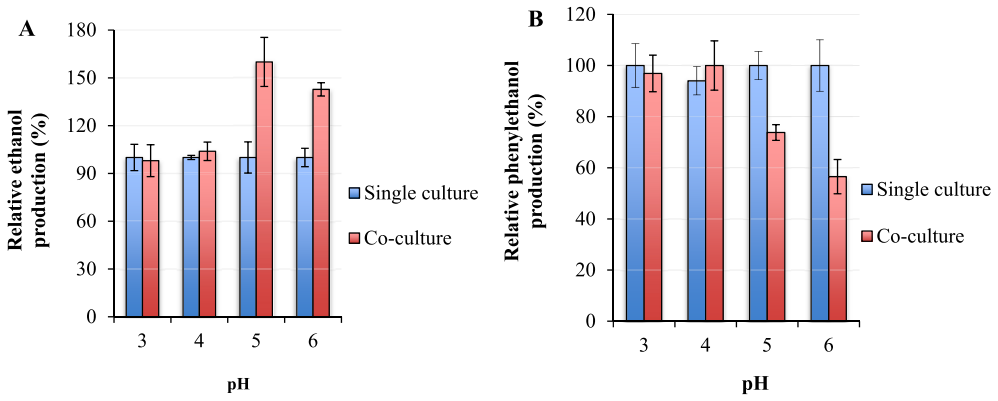

Figure 7. Ethanol production by *P. kudriazevii* in single and co-culture at different pH. A. Ethanol, B phenylethanol. Relative production was expressed as percentage, using the quantity of aroma produced in single culture as 100 %. Error bars indicate standard deviation between three replicates.

It was also observed that the quantity of volatile compounds (e.g., isoamyl acetate, phenylethanol, methylbutanol) produced by both strains in single culture did not increase in the co-culture treatment (Table 5). Figure 6 shows the level of volatiles production in single and co-culture. Similar modifications in volatiles production in co-culture were also observed at 35 °C, the optimum growth temperature for both strains (data not shown).

Since the pH was found to influence the interaction in co-culture during microbial growth, the productions of the main volatile compounds from *P. kudriazevii* YS201, notably ethanol and phenylethanol, were analyzed in various cultures differing in the medium pH. Figure 7 shows that, the production of both compounds was similar in single and co-culture at pH 3 and 4 whereas strong modifications in volatiles production in co-culture occur at pH 5 and 6.

4. Discussion

Interaction between two major microbial species notably *P. kudriazevii* YS201 and *B. subtilis* BS38, involved in Ivorian cocoa fermentation was investigated. Most of previous studies of microbiota from fermenting cocoa focused on strain identification and typing (Nielsen et al., 2007; Ouattara et al., 2017b), biochemical and functional properties (Pereira et al., 2013), and more recently the use of microbial cultures as starters in experimental cocoa fermentation (Leal et al., 2008; Meersman et al., 2016). Apart from the study describing an antifungal activity of a co-culture of yeast and lactic acid bacteria (Romanens et al., 2019), the direct interactions between different microbial species from fermenting cocoa have rarely been investigated. The present work provides new understanding of how microbial strains work together during fermentation of cocoa.

The pulp simulation medium (PSM) used in this study, although modified, allowed a good growth of the microorganisms analyzed that were able to reach an OD_{600} up to 10 for *P. kudriazevii* and 3 for *B. subtilis*, within 24 h. However, this absorbance corresponded to a relatively low count of viable cells that never reached 8 log (CFU/mL). In comparison to standards, an OD_{600} of 10 from yeast culture and 3 from *Bacillus* are expected to give respectively 8.47 and 9.17 log CFU/mL (Day et al., 2004; Fisher et al., 2001). Indeed many factors such as composition and pH of the medium are known to influence the OD of microbial culture.

Moreover, the maximum load of *Bacillus* and yeast obtained during natural fermentation of cocoa can sometime reach 9 log CFU/mL (Ardhana and Fleet, 2003; Nielsen et al., 2007; Schwan, 1998) that is higher than the growth obtained in PSM. This suggests that the PSM may be less favourable for microbial growth than the natural cocoa pulp. As to support this suggestion, we isolated in previous study *B. subtilis* strains throughout the fermentation process of cocoa even during the first stage of this process when pH was round 3.5 (Ouattara et al., 2008, 2011). Yet, in the present study, *B subtilis* BS38 as one of these strains was not able to grow in PSM at pH 3.5, and very poorly at pH 4.

Analysis of microbial growth at different pH in PSM shows that in co-culture, at pH 4, the yeast strain grew similarly to a single culture, and do not appear to be hindered by the presence of *Bacillus* that grow poorly indicating weak interaction. However, at pH 6 corresponding to optimum growth for both strains, a significant decrease of microbial population was observed for both strains growing in co-culture, indicating an antagonistic type of interactions. Such interactions could be responsible for differences in end products observed. For instance, phenylethanol and ethanol produced in single culture by *P. kudriazevii* YS201 did not differed significantly to that produced in co-culture at pH 3 and 4 at which *B. subtilis* BS38 had a poor growth. However, the remarkable variations in the metabolite production observed at pH 5 and 6 in co-culture may reflect strong interaction between strains. These interactions are also expected to occur at temperatures range in which both strains can grow well notably 35–40 °C as shown previous results (Samagaci et al., 2014; Yao et al., 2017). Such interactions leading to significant variations of metabolites production in microbial strains are undoubtedly critical since they could be exploited to increase certain metabolites of interest like ethanol and tune the quality of chocolate. However, these interactions could also present the disadvantage of lowering the level of certain aroma compounds such as phenylethanol as revealed this study.

As PSM may be less favourable for microbial growth than the natural cocoa pulp, results may not directly translate to the fermentation of cocoa. However, this may still provide insights into processes occuring in natural cocoa fermentation where microbial succession is characterized by a growth of yeast population to 9 log CFU/ mL during the 24 first hours, increasing pH from 3.5 to 4, while *Bacillus* load remains around 7 log CFU/ mL (Ardhana and Fleet, 2003; Pereira et al., 2012). This trend typically reverses in later stages of the fermentation when pH reaches 6, allowing *Bacillus* to reach up to 8 log CFU/mL while yeast decline to roughly 3 log CFU/mL (Ardhana and Fleet, 2003; Schwan, 1998). In this context, interactions are expected to occur during the period where both strains are present with relatively high load notably the first four days of cocoa fermentation (pH round 3.5–4.5 and temperature 30–35 °C) (Ardhana and Fleet, 2003; Schwan and Wheals, 2004; Kouame et al., 2015). According to Gibe and Pangan (2015) reported that the use of *Bacillus* and *Pichia* starter cultures proved to be efficient within this period of first four days. Another shortcoming of the PSM system used here, is the lack of lactic acid bacteria and acetic acid bacteria, which may alter how *Bacillus* and yeast interact in natural fermentation of cocoa.

Pulp-degrading enzymes are important to cocoa fermentation due to their ability to speed the fermentation process (Bhumibhamon and Jinda, 1997; Cempaka et al., 2014). Thin layer chromatography (TLC) was performed to further analyze the cooperative effect of Pel and PG and to define the contribution of each specific pulp-degrading enzymes. This study found that Pel and PG, at their respective optima pH, individually generated a mixture of different size oligomers from pectin degradation.

The absence of monomers among these pectin degradation products indicates that these enzymes lack exopectinase activity, and thus hydrolyze pectin randomly, leading to the same profile of end-products as shown by TLC. Unfortunately, it was not possible to check whether the chromatogram from combined action of Pel and PG is different from that of individual enzyme activity as these enzymes failed to degrade pectin at a same pH in liquid phase.

In contrast, synergistic degradation of pectin by these enzymes was observed on solid phase (pectinized plate agar and cucumber assays). These experiments may better mimic cocoa fermentation conditions, where pulp is the solid substrate from which water has drained at the beginning of the fermentation. This data also argues that enzymes activities measured from commonly used liquid phase may not accurately depict what is occurring during cocoa fermentation. It is generally reported that, surface adhesion-based culture such as solid-state fermentation (SSF) including microbial cells growing on a solid phase (biodegradable or not), produce more pectinase than culture in liquid medium (Submerged fermentation) (Kumar et al., 2011; Oumer and Abate, 2018). Our experiments show this may also be true for pectinase activity, shown here to be more efficient on solid phase (plate agar or cucumber) than in liquid. The cooperation of pulp-degrading enzymes from both strains *P. kudriazevii* YS201 and *B. subtilis* BS38 observed in this study, may also play a determinant role in speeding the fermentation process of cocoa. This is strongly supported by recent studies, reporting that a mixture of yeast and *Bacillus* species namely *Pichia mexicana* and *Bacillus pumilus* was successful in shortening fermentation process from 6 to 4 days while single treatment of either *P. mexicana* or *B. pumilus* was insufficient to ferment cacao beans within 4 days (Gibe and Pangan, 2015). Cucumber, as a standard model for studying the pulp-degrading capacity of enzymes (Schwan et al., 1997; Khan and Latif, 2016) proved to be suitable in our study since this cooperative effect of Pel and PG could not be revealed using the cocoa pulp itself.

Previously, it was reported that microorganisms could impact the aroma of the final chocolate by directly producing aroma compounds during fermentation of cocoa (Meersman et al., 2016). In the present study, aromas produced by *Pichia* in PSM belonged essentially to the class of alcohol namely ethanol, phenylethnol and methylbutanol and the class of esters notably ethyl acetate and isoamyl acetate. These compound were also found to be among the main aromas produced by yeast strain from fermenting cocoa, using saboureau medium as reported Koné et al. (2015), but without quantification of aroma compounds produced. Moreover, to the best of our knowledge, apart from a former study reporting the production of pyrazines (Zak et al., 1972), the volatile compounds production of *Bacillus* from fermenting cocoa, susceptible to modulate aroma quality of final chocolate has been rarely investigated. Here *B. subtilis* strain BS38 showed the ability to generate an array of aroma compounds, mainly acetoin, butanediol, methylbutanol and pyrazines. However, in co-culture, volatiles production by yeast decreased while *Bacillus* almost failed to produce its' potential of aroma compounds. This confirms the interactions between these strains in fermenting medium where volatiles from yeast should dominate the aroma from *Bacillus*.

The increase of ethanol production observed in co-culture during this study is indicative of a synergistic action of both strains. Likewise, *B. subtilis* and *Saccharomyces cerevisiae* were also used in co-culture to improve ethanol yield from wastewater (Tantipaibulvut et al., 2015). Generally, this synergy is thought to result from polysaccharides hydrolysis by *Bacillus* yielding metabolic substrates that are converted by yeast to ethanol. The synergy between *Bacillus* and yeast strain for ethanol production may play a crucial role since, this metabolite is highly required for a well processed cocoa fermentation (De Vuyst and Weckx, 2016).

In conclusion, this study evidenced interactions between two microbial species during fermentation of cocoa pulp and suggests the probable existence of other interactions between other microorganisms in the fermenting cocoa. It also opens new perspectives of research on microbial interactions that may help for better understanding of the exact role of the microbiota in fermenting cocoa and for better manipulation of starter strain to improve this fermentative process.

Declarations

Author contribution statement

Honoré G. Ouattara: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Ryan J. Elias: Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Edward G. Dudley: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

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