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A preliminary study on a novel bioaugmentation technique enhancing lactic acid production by mixed cultures fermentation

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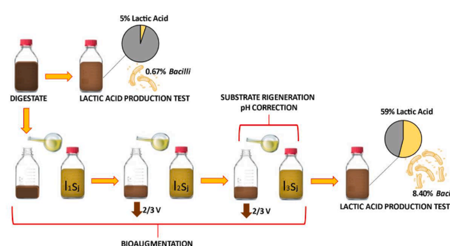
HIGHLIGHTS

- A bioaugmentation technique to select cultures producing lactic acid is proposed.
- The selection occurs via sequential batch steps with intermittent pH correction.
- Substrate concentration affects the efficacy of the bioaugmentation technique.
- As consequence of the bioaugmentation, lactic acid increases from 5% to 59%
- The technique results in microbial population change to *Bacillus* species.

GRAPHICAL ABSTRACT



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ABSTRACT

The paper is a preliminary study on the selection of lactic acid producing microorganisms from a mixed microbial population via bioaugmentation. The bioaugmentation technique is based on pH sudden variations occurring in sequential batch steps of a dark fermentation process applied to simple substrates. Different conditions are tested and compared. The structure of microbial communities and concentrations of metabolic intermediates are analyzed to study the possible substrate conversion routes. Obtained results indicate that the initial mixed culture produced a lactic acid percentage of 5% in terms of $COD_{LA}/COD_{PRODUCTS}$. In the most favourable conditions, the selected culture produced a lactic acid percentage of 59%. The analysis of the composition of microbial communities before and after the bioaugmentation processes, indicates that lactic acid production mainly results from the population change to bacteria belonging to the genus *Bacillus*. Indeed, the relative abundance of *Bacilli* increased from 0.67%, to 8.40% during the bioaugmentation cycle.

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1. Introduction

The development of new strategies for an efficient production of lactic acid (LA) is of great interest, due to the wide application of this product in chemical, pharmaceutical and food industries (Luongo et al., 2019). Moreover, LA can be used to produce a polymer (polylactic acid) able to replace petrochemical plastics in several applications (Liang et al., 2015). This is why LA market is supposed to reach 9.8 billion USD by 2025 (Rombouts et al., 2020), with an annual growth rate of almost 19% (Oliveira et al., 2020).

Among different LA production alternatives, microbial fermentation is gaining increasing attention, being the less expensive and the most environmental friendly solution (Rawoof et al., 2021).

Currently, pure bacterial fermentations account for 90% of LA production worldwide (Oliveira et al., 2020), although the use of mixed cultures would be more attractive, because of its intrinsic economical and operational advantages, including the possibility of using waste material as bacterial substrates, with no need of sterilisation (Regueira et al., 2021). Indeed, the use of mixed culture fermentation for LA production is still challenging to date. Recently, it has been reported that LA may result as the dominant product of mixed microbial fermentations if specific substrates are used. Rombouts et al., (2020) studying mixed fermentation for LA production, observed that the microbial community could be dominated by lactic acid bacteria (LAB) (e.g. *Lactococcus* and *Lactobacillus* species) using a rich medium containing peptides and B vitamins. On the contrary, employing a simple mineral medium, the substrate is mainly fermented to acetate and butyrate by species belonging to the *Clostridia* class.

Luongo et al., (2019) observed that indigenous cultures of cheese whey allow obtaining LA as the main fermentation product. At the same time, the authors proved that the use of an external inoculum for LA production from cheese whey at uncontrolled pH, resulted in acetic acid and ethanol production. The study also demonstrated that LAB are able to grow at extremely low pH (Luongo et al., 2019). The external pH decrease do not constitute an acid shock as LAB are naturally able to acidify the external medium (Siegumfeldt et al., 2000). Moreover, LAB are more acid-tolerant than other fermentative bacteria (Padmavathi et al., 2018; Wang et al., 2018). Consequently, various researchers adopted acidic pre-treatments to favour LAB proliferation in mixed culture fermentation. This procedure limited the production of other catabolites, and increased the lactic acid percentage in the fermentation broth (Choi et al., 2016; Itoh et al., 2012).

Generally, a rapid change of the external pH represents a stressful situation for microorganisms. However, differently from most non-lactic acids producing bacteria, LAB are able to regulate their intercellular pH as an adaptation strategy (Siegumfeldt et al., 2000). When the external pH decrease, neutrophilic bacteria maintain an internal pH that is close to 7. The high internal-external pH difference generates large proton gradients, which is disadvantageous for fermentative bacteria. Indeed, proton translocation requires energy (Kobayashi et al., 1986). On the other hand, many acid-tolerant microorganisms, such as LAB, have developed a different strategy: The internal pH decreases as the external pH decreases to maintain a constant pH gradient rather than a constant internal pH (Siegumfeldt et al., 2000). Several possible methods which can be used by bacteria to regulate the internal pH have been studied, such as the synthesis of cytoplasmic buffer, proton symport systems, production of acid or bases and proton pumps (Hutkins and Nannen, 1993).

Akao et al. (2007) showed that acidic pre-treatments are able to promote the selection of LAB from mixed cultures, although, in that sense, even better results can be obtained through a swing pH control of the system (Tashiro et al., 2016).

Starting from these results, the present paper proposes a preliminary study on an innovative operative strategy to improve the selection of LAB from an external inoculum, in order to maximize LA production from a simple medium. The proposed strategy, indicated as bioaugmentation cycle, is based on the development of three sequential fermentative steps, conducted in batch conditions. The selection of LAB simply occurs as consequence of pH variations, induced by the applied organic charge and by the punctual pH corrections effectuated at the beginning of each step. To optimize the bioaugmentation efficiency, various Food to Microorganisms (F/M) ratios are tested. No nutrient addition is operated, as the study is aimed at developing a technique which could be applied in the presence of simple substrates. Therefore, the present study represents an indication to address future researches aimed at producing lactic acid from both unrelated substrates and external inocula, acting on operational conditions only.

Table 1
Operative conditions of fermentative steps (I_iS_j).

Cycle #	Step 1	Step 2	Step 3	F/M ratio (mgCOD/mgCOD)	Substrate concentration (g/L)
1	I_1S_1	I_2S_1	I_3S_1	0.025	0.60
2	I_1S_2	I_2S_2	I_3S_2	0.5	1.20
3	I_1S_3	I_2S_3	I_3S_3	0.1	2.40
4	I_1S_4	I_2S_4	I_3S_4	0.25	6.01
5	I_1S_5	I_2S_5	I_3S_5	0.5	12.03

2. Materials and methods

2.1. Experimental apparatus and materials

Experimental tests were conducted in batch mode, at mesophilic temperature (35 ± 1 °C), using 500 mL glass reactors (400 mL working volume). The reactors were equipped with two different ports, used for liquid and gas sampling operations. Plastic tubes and gaskets were used for junctions. Before use, sealing joints were controlled, filling each reactor with water and pressurized air. High purity chemicals were used for substrate preparation and all analytical determinations. Adopted glassware were soaked overnight in a nitric acid bath (2% v/v) and rinsed several times with bi-distilled water.

2.2. Bioaugmentation cycle

The bioaugmentation cycle was composed by three successive fermentative steps, each conducted using, as inoculum ($I_{i=1\text{-}3}$), the biomass selected in the previous step. The external mixed culture, used as initial inoculum (I_1), was sampled from a full-scale anaerobic treatment plant located in Casal di Principe (South of Italy), processing the organic fraction of municipal solid waste. The characteristic of the digestate were: COD = 77.30 ± 0.08 g/L, pH = 8. Before use, the inoculum was pre-treated by heat shock, as detailed described elsewhere (Luongo et al., 2019). Used substrate for each fermentation step was a synthetic glucose solution, prepared dissolving the solid compound in bi-distilled water.

Five different operative conditions were tested, corresponding to five different initial concentrations of the substrate ($S_{j\,=\,1\text{–}5}$), as summarized in Table 1. Substrate concentrations were calculated setting different initial F/M ratios. Each condition was conducted in triplicates. For simplicity, experiments are reported as I_iS_j, where I = inoculum and i = 1–3 is the step number. S = substrate and j = 1–5 represent the five different substrate concentrations.

The reactor adopted for the first step was filled with the external inoculum, and the substrate maintaining a 1:2 volumetric ratio (v/v). The reactor was operated until no increase in terms of lactic acid production was detected. The obtained fermented mixture was used as inoculum of the second fermentative step. This latter was performed keeping the same volumetric ratio of the previous step between inoculum and substrate, and the same substrate concentration. The reactor was operated in the new conditions until no increase in terms of lactic acid production was detected. Once more the obtained fermented

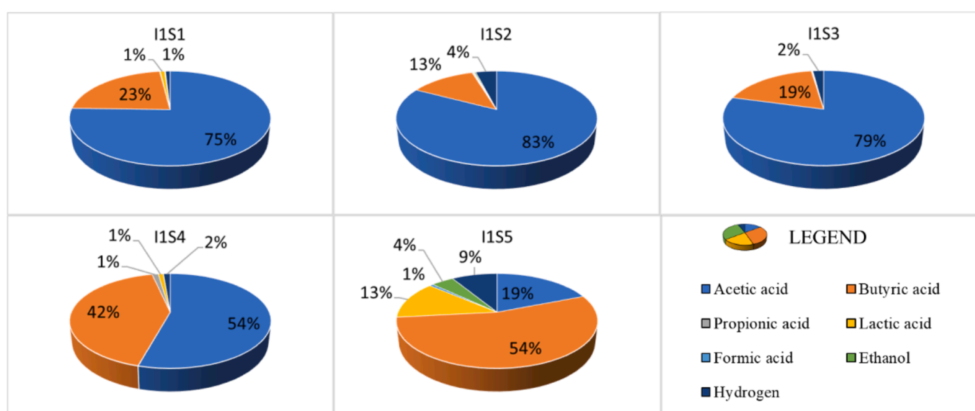

Fig. 1. Fermentation products of the first step (I_1S_j) of the bioaugmentation cycles.

mixture was used as inoculum of the subsequent and last step, keeping the volumetric ratio between inoculum and substrate equal to 1:2. At the beginning of each step the pH value was adjusted to 6.0 ± 0.01, using a 1 M NaOH solution. No pH correction was operated, instead, during the fermentation period. At selected time (24 h), organic acids (OAs) concentration, hydrogen production and pH variation were measured. Moreover, a microbial characterization was conducted on the initial inoculum and on the fermented mixtures obtained at the end of the bioaugmentation cycle.

2.3. Lactic acid production tests

The fermented mixtures produced at the end of the five bioaugmentation cycles corresponding to the five tested F/M conditions, were used as inoculum for LA production tests (I-$PT_{j=1-5}$). The fermented mixture from the reactor I_3S_1 was used as inoculum for the test I-PT_1 and so on. LA production tests were conducted using once more glucose as substrate, keeping the volumetric ratio between inoculum and substrate equal to 1:2 (v/v). The substrate concentration was fixed to 24.06 g/L in order to compare the performances of the different cycles adopting the same operative conditions. A blank test (I-PT_0) using, as inoculum, the initial mixed culture (I_1) was performed too. In all cases, the initial pH was adjusted to 6.0 ± 0.01. At selected time (24 h), OAs concentrations, hydrogen production and pH variation were measured.

2.4. Analytical methods and instruments

OAs concentration was determined by high-pressure liquid chromatography (HPLC), using a LC 25 Chromatography Oven (Dionex, USA) equipped with an Organic Acids column (Metrohom, Switzerland) and an UVD 340U detector (Dionex, USA). pH was measured using an inoLab pH meter (WTW, Germany). COD concentration was measured by colorimetric analysis, according to the Standard Methods (APHA, 2005). Biogas quantitative determination was performed by water displacement, according to Policastro et al., (2020). Biogas composition was successively analyzed by gas chromatography, using a Varian Star 3400 gas chromatograph equipped with Shin-Carbon ST 80/100 column and a thermal conductivity detector.

The extraction of the total DNA was carried out to sequence the genome of the whole microbiota, employing the Next Generation Sequencing (NGS) technology, targeting bacterial 16S rRNA gene. For each sample under analysis, 10 g were aliquoted and centrifuged to extract DNA from the supernatant. Supernatant was transferred into sterile 2 mL vials containing 0.5 g glass beads. CTAB extraction protocol (Doyle, 1991) was carried out to recover total DNA from the samples. The extracted DNA samples were amplified with PCR using the V3 and V4 primers (V3: TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG; V4: GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC),

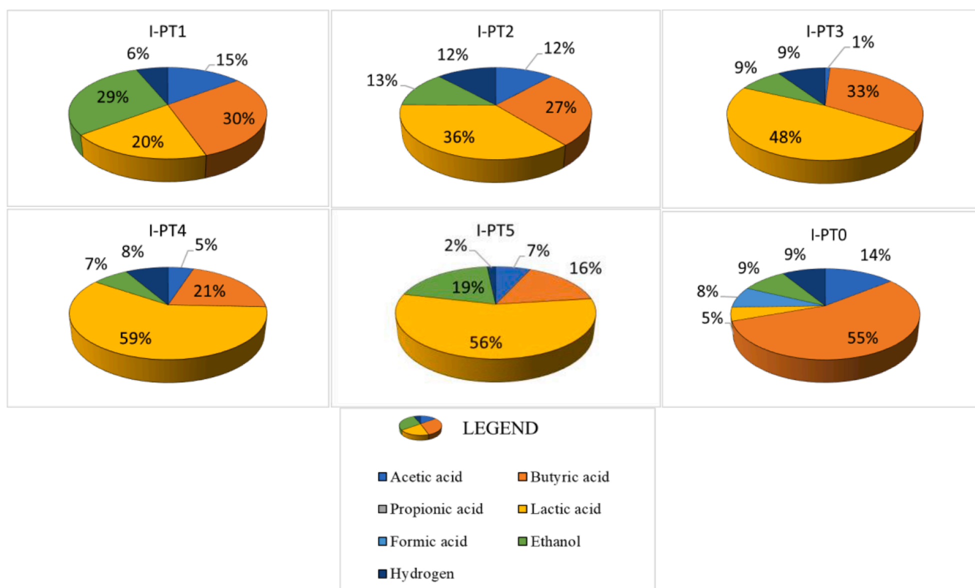

Fig. 2. Fermentation products of the lactic acid production tests (I-PT_j).

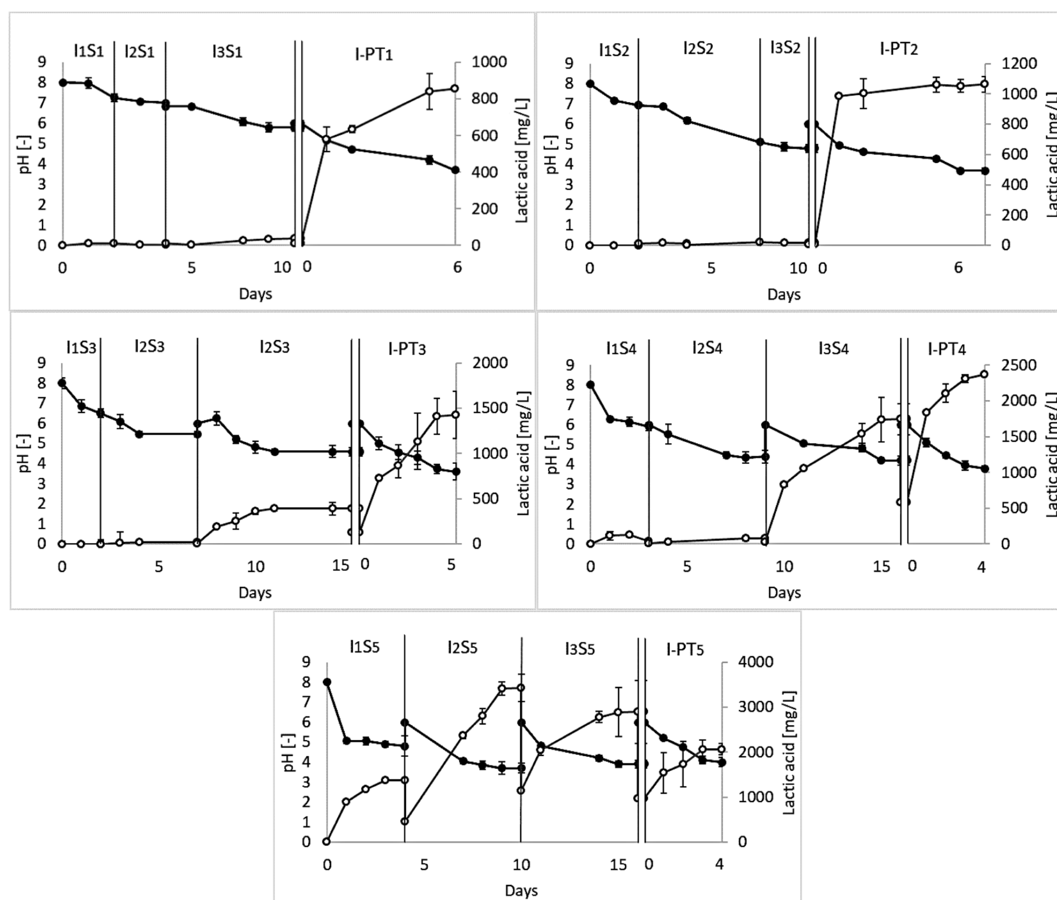

Fig. 3. Lactic acid concentration and pH trend during the 3 steps of the bioaugmentation cycle (I_iS_j) and the final LA production tests ($I\text{-}PT_j$).

complementary to V3-V4 variable region of the bacterial 16S rRNA gene (500 bp). Sequencing was conducted with a MiSeq Illumina platform, using 2 × 300 bp paired end, 600 cycles, following the manufacturer's instructions (Illumina MiSeq, USA). Differences in the group's communities retrieved from Illumina experiment were assessed by anosim using weighted UniFrac distance, and Anova using Bray Curtis distance (Mothur) (Corniello et al., 2021).

3. Results and discussion

Figs. 1 and 2 report the percentages of fermentation products (i.e. OAs and hydrogen) calculated as ($COD_{SINGLE\ PRODUCT}/COD_{TOTAL\ PRODUCTS}$)*100, obtained during the first step of the bioaugmentation cycle ($I_1S_{j=1-5}$) and the LA production tests ($I\text{-}PT_{j=0-5}$). The OAs percentages reported for the LA production tests represent the net productions.

The obtained results indicate that acetic acid (A) and butyric acid (B) were the main soluble products of the fermentation process, during the first step of the bioaugmentation cycles. Such results were in agreement with previous studies on mixed cultures fermentation processes (González-Cabaleiro et al., 2015; Khanal et al., 2004). In particular, acetic acid was prevalent in I_1S_1, I_1S_2, I_1S_3 and I_1S_4 sets, while butyric acid was prevalent at higher glucose concentrations. During the LA production tests, a strong decrease of the acetic acid and butyric acid concentrations occurred. Moreover, the ratio between butyric and acetic acid (B/A) increased during these tests, compared to the first step of the bioaugmentation cycles. No propionic acid was ever detected. Probably, the heat shock pre-treatment might have inhibited non-spore-forming propionate producers (Ghimire et al., 2015). On the other hand, ethanol production was almost negligible in the first step of the bioaugmentation cycles (except I_1S_4 set) and increased in the LA production tests, suggesting that microorganisms principally followed the heterolactic fermentation pathway (Rombouts et al., 2020).

In terms of biogas production, no methane was ever detected. Conversely, hydrogen was produced both during the I_1S_j steps and during the $I\text{-}PT_j$ tests. Hydrogen yield was strongly related to the presence of butyric acid. A general reduction of both hydrogen production and butyric acid was observed during the LA production tests, principally for cultures selected at higher glucose concentrations.

Lactic acid production was observed only in I_1S_3 reactor. However, in the $I\text{-}PT_j$ tests, all sets of reactors produced a considerable percentage of lactic acid as a result of the bioaugmentation technique. The most efficient set was the $I\text{-}PT_4$ one, suggesting that the adopted condition was the most appropriate for lactic acid bacteria selection. The $I\text{-}PT_4$ set produced the higher lactic acid percentage (59%) corresponding to 0.51 $g_{LA}/g_{CONSUMED\ GLUCOSE}$. The only exception was represented by the blank test ($I\text{-}PT_0$), which produced a low amount of lactic acid (5%) corresponding to 0.04 $g_{LA}/g_{CONSUMED\ GLUCOSE}$.

Fig. 3 reports the lactic acid and the pH trends during the three steps of the bioaugmentation cycles (I_iS_j) and during the LA production tests ($I\text{-}PT_j$).

The initial pH value was 8 for all the experimental sets. During the first step, the conversion of glucose to OAs generated a pH drop. As expected, the higher the glucose concentration the more the pH dropped. pH decrease varied from 10% (I_1S_1) to 50% (I_1S_5). Lactic acid was not detected in I_1S_1, I_1S_2 and I_1S_3 reactors. On the other hand, it was produced in the I_1S_4 and I_1S_5 sets, which were the only ones that reached a pH lower than 6. Therefore, at the end of the first step, the pH correction to 6 was performed only for sets I_1S_4 and I_1S_5. During the second step, a slight pH decrement was observed for reactors I_2S_1, I_2S_2 and I_2S_3. However, lactic acid production was not significant. The same

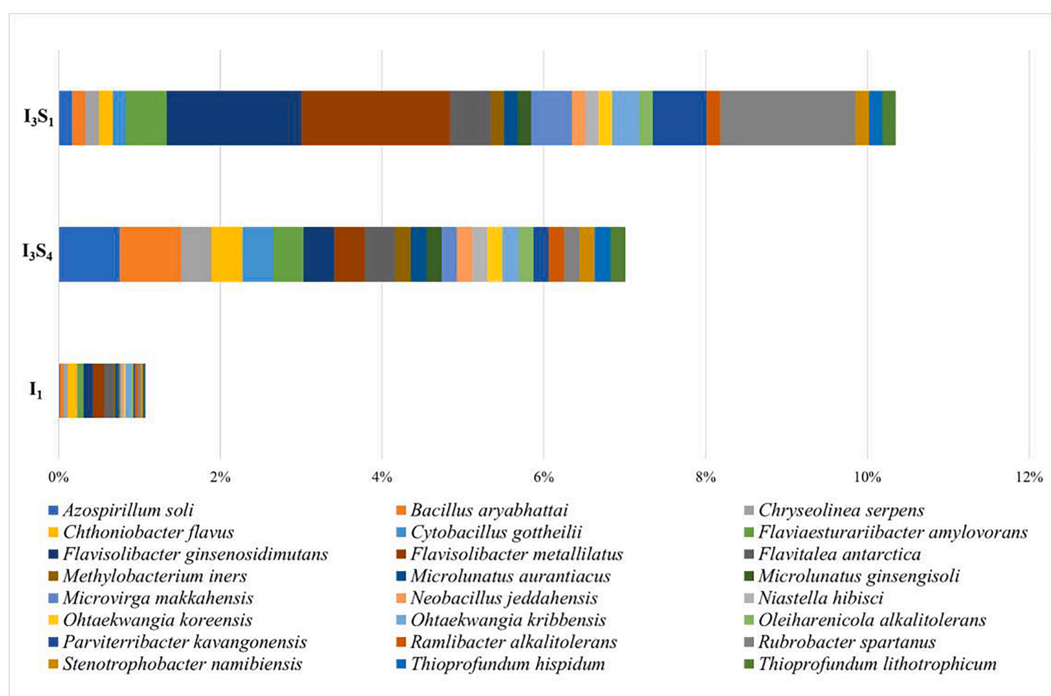

Fig. 4. Barplot at species level analysing common microorganisms detected in samples I_1, I_3S_1 and I_3S_4.

result was observed for reactor I_2S_4. Reactors I_2S_4 did not produce significative lactic acid amounts, even though the pH dropped from 6 to 4.8. On the other hand, I_2S_5 produced a lactic acid amount which was more than doubled with respect to the first cycle. At the end of the second cycle, the pH correction was performed for reactors I_2S_3, I_2S_4 and I_2S_5. At the third step, a relevant increase of the lactic acid production was observed for reactors I_3S_3 and I_3S_4, while a reduction was detected for I_3S_5. As for reactors I_3S_1 and I_3S_2, the lactic acid concentrations were registered mainly at the same not significant values of the previous steps. At the end of the third step, the pH of all reactors was lower than 6: thus, pH correction was applied within all sets. During the LA production tests, lactic acid production increased in all sets of reactors. A concomitant pH drop was observed. However, the production of the I-PT_5 set was lower, compared to that observed during the two previous steps of the bioaugmentation cycle. Such results suggest that lactic acid production was related to sudden pH changes more than to an acidic pH environment. Indeed, before the first pH correction, only when pH decreased suddenly, it was sudden produced lactic acid. Otherwise, the most significative lactic acid increase was observed after the first pH correction. To support this hypothesis, control experiments were performed at uncontrolled pH. In this case, lactic acid production was significative only during the I-PT_j tests, when the glucose concentration was high enough to cause a rapid accumulation of acids and, therefore, a rapid pH drop (data not shown). These results agree with previous studies reporting that acidic conditions are able to promote the selection of LAB from mixed cultures, even though, better results can be obtained through a swing pH control of the system (Tashiro et al., 2016).

To control the change of the microbial community due to the bioaugmentation cycle, microbial composition analysis was performed. The analysis was conducted on the fermented mixtures sampled from reactors I_3S_1 and I_3S_4 (used as inoculum respectively for reactor I-PT_1 and reactor I-PT_4) and the initial inoculum. The selected fermented mixtures corresponded to the bioaugmentation cycles, which resulted more performant during the LA production tests.

Among the most abundant species detected in the initial inoculum, 3 Firmicutes phylotypes and 17 Euryarchaeota phylotypes were identified. Firmicutes phylotypes were related to bacteria in the classes of Clostridia and Tissirellia. The 17 Euryarchaeota phylotypes belonged to Methanomicrobiaceae, Metanotrichaceae, Methanospirillaceae and Methanoregulaceae Families. Methanomicrobiaceae, Methanospirillaceae and Methanoregulaceae are known to as methanogens, exploiting H_2/CO_2 or formate to produce methane (Garrity et al., 2001; Qiao et al., 2013). Hence, microorganisms belonging to the Methantrichaceae Family, resulting most dominant in the analysed samples, are classified as acetoclastic methanogens (Qiao et al., 2013). The microbial composition of the initial inoculum is in agreement with previous analysis of anaerobic digestion effluents (Qiao et al., 2013).

Bacterial communities resulting from the two bioaugmentation cycles showed higher diversity at Phylum and Genus level compared to the initial inoculum. Indeed, Firmicutes, Actinobacteria, Bacteroidetes, and Proteobacteria were found to be abundant in both I_3S_1 and I_3S_4, while they were not dominant in the initial inoculum. Notably all the most abundant species contained in the selected fermented mixtures were different compared to the initial inoculum, confirming the effectiveness of the bioaugmentation technique. The changes in the relative abundance of the most common species detected in all the three different samples are reported in Fig. 4.

The relative abundance of all the studied species increased during the bioaugmentation cycle. In particular, *Azospirillum soli, Bacillus aryabhattai, Chryseolinea serpens, Chthoniobacter flavus* and *Cytobacillus gottheilii* were more abundant in the I_3S_4 sample compared to the I_3S_1 one. Conversely, *Flaviaesturariibacter amylovorans, Flavisolibacter ginsenosidimutans, Flavisolibacter metallilatus, Flavitalea Antarctica, Ohtaekwangia kribbensis, Microvirga makkahensis, Parviterribacter kavangonensis and Rubrobacter spartanus* were present in higher relative abundance in the sample I_3S_1, compared to the I_3S_4 one. The remaining species were detected in similar percentages in both samples. It is worth noting that the abundance of species belonging to the genus bacillus was higher in the I_3S_4 sample. Comparing microbiological results obtained in this study with other works on lactic acid production by mixed cultures, it can be stated that acidic pre-treatments operated at constant pH are efficient when LAB such as species from *Lactobacillus*, *Lactococcus* or *Pediococcus* genera are present in the culture (Akao et al., 2007; Juodeikiene et al., 2016). In this study, these species were absent in all the analyzed samples. However, the composition of the microbial cultures selected in this study was similar to those reported in other studies on

fermentation processes producing lactic acid (Choi et al., 2016; Xu et al., 2018).

Considering all microorganisms detected in the three samples, the phylotypes that contribute to the presence of genes encoding the enzymes for lactate generation were *Bacillus* and *Corynebacterium*. The percentages of the genus *Bacillus* were: i) 0.67% in the initial inoculum; ii) 8.40% in the I_3S_4 sample; and iii) 1.77% in the I_3S_1 sample. The percentages of the genus *Corynebacterium* were, instead: 0.74% in the initial inoculum; ii) 0.94% in the I_3S_4 sample; and iii) 0.83% in the I_3S_1 sample.

3.1. Effect of the substrate concentration and the bioaugmentation technique on the fermentation process

Despite the abundant presence of methanogens, no methane was detected during the first step of the bioaugmentation cycle, demonstrating the efficiency of the heat shock pre-treatment. In general, the aim of inoculum pre-treatments is to create extreme conditions which are favourable to spore-forming fermentative bacteria, and hostile to non-spore-forming methanogens (Kraemer and Bagley, 2007; Luo et al., 2011). Of course, methanogens are only temporarily inhibited by the pre-treatment. Usually, in long-term processes (e.g. continuous fermentations), methanogenic archaea populations re-establish their activity (Luo et al., 2011). In this study, the absence of methanogens in I_3S_1 and I_3S_4 samples and the absence of methane production in the LA production tests demonstrate that the bioaugmentation technique was effective for the methanogens wash out. The results were consistent with the high sensitivity of methanogens to pH values (LEITAO et al., 2006).

The prevalence of acetic acid and butyric acid in reactor I_1S_j indicates that microorganisms principally followed the acetate and butyrate pathways, rather than a mixed fermentation pathway. Moreover, as reported in previous studies, the fermentative metabolism of Clostridium species, which were abundant in the initial inoculum, produces mainly butyrate and acetate as primary soluble metabolites (Lin et al., 2007).

The observed dominance of the butyric acid pathway at increasing glucose concentration, also reported by (García-Becerra et al. 2019) probably indicates a stress condition due to the pH drop. Indeed, being glucose a rapidly biodegradable sugar, its bioconversion to hydrogen and organic acid was fast.

Very peculiar are the results related to hydrogen production. Usually, hydrogen yield increases at increasing B/A ratios (Hawkes et al., 2007). In this study, however, hydrogen production is more strongly related to the presence of butyric acid rather than to the B/A ratio. From a theoretical point of view, the metabolic route from glucose to acetate produces higher hydrogen yields compared to the pathway from glucose to butyrate (Ghimire et al., 2015). However, acetate can also be a product of hydrogen consumers, such as homoacetogens belonging to the *Clostridium* genus. Since homoacetogenesis can occur concomitantly with biohydrogen production, such an event might have promoted hydrogen consumption to produce acetate (García-Becerra et al., 2019; Siriwongrungson et al., 2007). Conversely, the butyrate pathway is inevitably linked to hydrogen production in mixed culture (Guo et al., 2014). Due to the microbial selection, a lower acetic acid concentration was detected in the LA production tests, indicating a limited acetogenesis. This hypothesis is also supported by the higher B/A ratios observed in I-PT_j tests compared to the I_1S_j ones.

During the I_1S_i step, lactic acid production was observed only in reactors operated at high substrate concentrations. This result was attributed to the sudden pH drop, due to the higher initial glucose concentration. Nevertheless, lactic acid was detected in all the I-PT_j tests, suggesting that a shift to lactate production pathway occurred during the selection cycles. As previously mentioned, the concomitant presence of ethanol may indicate that microorganisms principally followed the heterolactic fermentation pathway (Rombouts et al., 2020).

3.2. Influence of the pH on lactic acid production

As mentioned before, the analysis of the pH and lactic acid trends lead to the evidence that lactic acid production was related to sudden pH changes more than to an acidic pH environment. The most efficient set was the I-PT_4 one, suggesting that the adopted condition guaranteed the most appropriate pH oscillation. The bioaugmentation technique operated at lower substrate concentrations was not efficient enough, while the higher glucose concentration of 12.03 (corresponding to the initial F/M ratio of 0.5) led to the progressive inhibition of the overall microbial community.

Among the microorganisms detected in the analyzed samples, the phylotypes that contribute to the presence of genes encoding the enzymes for lactate generation were *Bacillales* and *Corynebacteriales* (Mugnai et al., 2021; Ohara and Yahata, 1996; Poudel et al., 2016; Tashiro et al., 2016). *Corynebacteriales* relative abundance kept at almost the same percentages in I_3S_1, I_3S_4 an I_1 samples, suggesting that they were not affected by the bioaugmentation technique. Therefore, *Corynebacteriales* could have partially contributed to the lactic acid production. Conversely, microorganisms belonging to the genus *Bacillus* were present in higher relative abundance after the selection. In particular, the higher percentage was detected in the I_3S_4 fermented mixtures, which showed the best performances in term of lactic acid production. Most likely, the pH variation obtained using a substrate concentration of 6.01 g/L (corresponding to an initial 0.25F/M ratio) and intermittent pH adjustments determined a selection of lactic acid producing bacteria belonging to the *Bacillus* genus. Results also suggest that the pH oscillation was beneficial for *Bacillus* growth, more than a less variable acidic pH.

Compared to other lactic acid producing bacteria, *Bacillus* species have several advantages that could have helped their selection and proliferation in the conditions adopted in this study. Indeed, literature studies report that *Bacillus* species can grow and produce lactic acid using poor media instead of nutrient-rich and expensive media (Wang et al., 2011). Moreover, they are able to produce lactic acid at both low and/or high pH (Abdel-Rahman et al., 2013). Such characteristics are also advantageous in terms of process management and costs. Indeed, the risk of the contamination during the fermentation is reduced and the costs related to nutrients and sterilization are avoided. Moreover, as reported by other authors, it is also possible that the other fermentative species had a metabolic change to the lactic acid production pathway (Guo et al., 2014; Lin et al., 2007). Indeed, (Lin et al., 2007) showed that when glucose was limiting, Clostridium butyricum shifted from the acetate/butyrate pathway to the lactate/ethanol one. Even when glucose was further supplied, the metabolic routes did not return to initial production pathways. The metabolic shift could have been facilitated by the present of glucose (García-Becerra et al., 2019). Indeed, under sugars availability, an accumulation of the intermediate pyruvate can occur. Pyruvate conversion to lactate represent a single step transformation that channels the surplus of electrons generated by fermentable sugars availability besides yielding reducing power to fermentative microorganisms (Elbeshbishy et al., 2017). Moreover, Corcoran et al., (2005) observed that the inclusion of carbohydrates which could be utilized by *L. rhamnosus* GG resulted in enhanced survival, while the survival effect was lost in cultures containing nonmetabolizable sugars, thereby establishing a relationship between glycolysis and enhanced survival in acidic conditions.

Chemical results reported in this study are in agreement with previous studies, performed on operating conditions of mixed culture fermentation processes. Temudo et al., (2007) investigated the influence of operational conditions on open mixed culture fermentation and observed that lactic acid was detected in higher concentrations during transient states, such as a rapid increase of the substrate concentration, changes of the dilution rate or pH variations. Moreover, Sakai et al., (2000) observed that the intermittent pH neutralization led to a stable and reproducible lactic acid production and inhibited the non-lactic acid

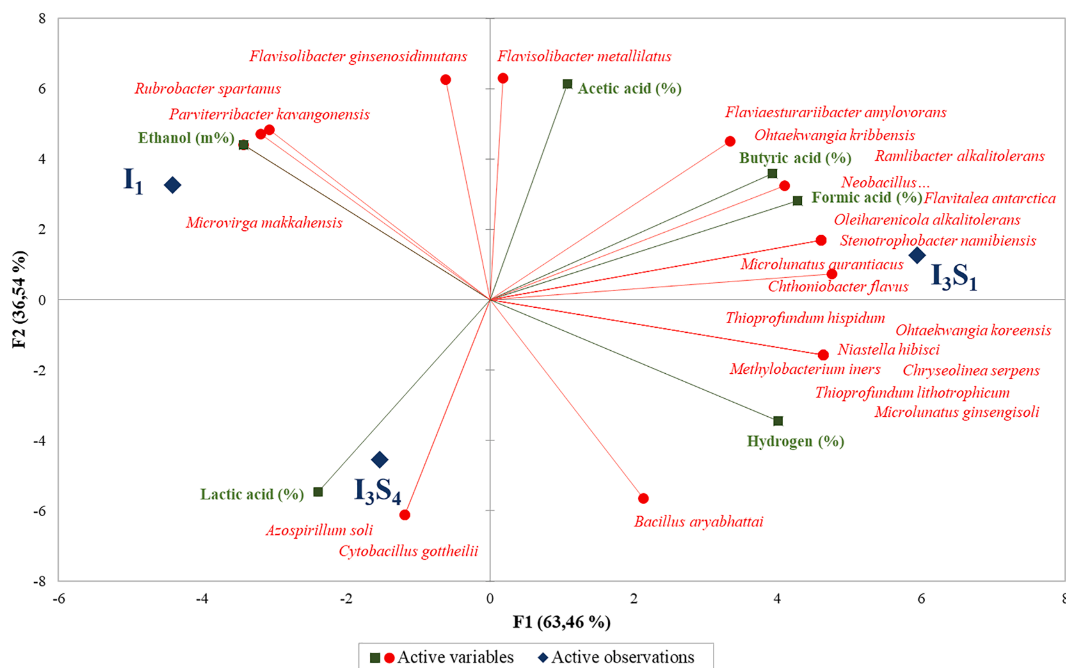

Fig. 5. Principal Component Analysis (axes F1 and F2: 100,00%).

bacteria growth. Finally, Tashiro et al., (2016) demonstrated that the pH constant control throughout fermentation did not improve lactic acid production. Conversely, a pH control strategy consisting in a switch from swing control to constant control promoted the lactic acid production pathway.

All described results are congruent with the Biplot diagram reported in Fig. 5, representing the 2D representation of the Principal Component Analysis, generated employing the data set corresponding to the LA production tests I-PT$_0$, I-PT$_1$ and I-PT$_2$.

As an example, according to the directions of the microorganisms' vectors, and considering measured chemical parameters, LA production may depend on *Bacillus* sp., *Cytobacillus* sp., *Azospirillum* sp.

4. Conclusions

The study represents a first step on the development of a novel bioaugmentation technique for the selection of mixed cultures producing lactic acid. The technique may be applied to external inocula, and to any waste substrate rich in fermentable carbohydrates, without additional requirements in terms of macro and micro-nutrients availability.

The technique guarantees a pH oscillation, that enhance the selection of mixed microbial cultures producing lactic acid. The metabolites and microbial community analysis suggest that lactic acid production results from the population change to lactic acid producing bacteria belonging to the genus *Bacillus* and/or a metabolic shift of fermentative bacteria.

CRediT authorship contribution statement

Grazia Policastro: Conceptualization, Investigation, Data curation, Methodology, Formal analysis, Visualization, Writing - original draft, Writing - review & editing. **Federica Carraturo:** Methodology, Data curation, Formal analysis, Writing - review & editing. **Mariacristina Compagnone:** Methodology, Investigation, Writing - review & editing. **Marco Giugliano:** Investigation, Data curation, Methodology, Formal analysis. **Marco Guida:** Supervision, Resources, Writing - review & editing. **Vincenzo Luongo:** Conceptualization, Writing - original draft, Writing - review & editing. **Raffaele Napolitano:** Investigation, Data curation, Methodology, Formal analysis. **Massimiliano Fabbricino:** Conceptualization, Supervision, Resources, Writing - original draft, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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