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Bio-acidification of animal slurry: Efficiency, stability and the mechanisms involved

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ABSTRACT

Slurry acidification is effective for reducing gaseous emissions during slurry storage. However, an alternative to sulfuric acid traditionally used is needed. This study investigated the efficiency of slurry bio-acidification treatment by adding different types and amounts of fermentable substrates to initiate and sustain the fermentation process. The carbon pools in the slurry were quantified to understand the mechanisms involved during the bio-acidification. Substrate addition efficiently reduced slurry pH during storage via lactic acid production. Substrates with a low pH proved beneficial in initiating the fermentation process, but higher glucose dosage did not produce the highest lactic acid concentration. Once the treated slurries reached pH 4.2 during the fermentation process, the production of lactic acid was promoted and provided substrate was still available, the weaker volatile fatty acids were avoided, resulting in lower CH_4 emissions. In conclusion, bio-acidification could replace the sulfuric acid to reduce gaseous emissions during slurry storage.

1. Introduction

Animal slurry is an important source of ammonia (NH_3) and greenhouse gas (GHG) emissions, contributing 80 % and 9.5 % of total NH_3 and GHG emissions respectively (Petersen et al., 2012; Gerber et al., 2013). These emissions are released during all steps in the slurry management chain, from production and storage to field application (Overmeyer et al., 2021; IPCC, 2019). Slurry is normally stored for several months before it can be applied to agricultural fields and in this time significant amounts of these gases are released to the atmosphere (Kupper et al., 2020), resulting in serious environmental and climate impacts. It is therefore crucial to reduce these emissions in order to protect the environment and human and animal health, and increase the nutrient value of the slurry by retaining nutrients otherwise lost to water and air.

Traditional acidification of slurry using sulfuric acid (H_2SO_4) reduces NH_3 volatilisation by up to 70 % by lowering the pH to 5.5, and has also been proven to reduce methane (CH_4) emissions by up to 87 % (Petersen et al., 2012). Acidification using H_2SO_4 is a cost-effective abatement technology that increases the slurry fertiliser value as more NH_4^+ is kept in the slurry in its dissolved form (Conn et al., 2007). Acidification with H_2SO_4 has been applied commercially in Denmark at farm scale since 2003 using automatic dosing systems in either slurry storage tanks or field application equipment. In 2014, approximately 18 % of all Danish animal slurry was acidified (Vestergaard, 2014).

However, even though H_2SO_4 is one of the cheapest inorganic acids on the market, the treatment is still a cost since between 5 and 7 kg of acid per tonne of slurry is required to decrease the pH to 5.5 or 6, depending on the slurry's composition and buffer capacity (Petersen et al., 2014; Kai and Pedersen, 2008).

Additionally, acidified slurry using H_2SO_4 cannot be used as a fertiliser in organic farming (Hjorth et al., 2015a). The use of synthetic acids is prohibited on organic farms under current EU and national organic certification schemes, and these farms also need to reduce their NH_3 emissions and increase their slurry fertiliser value. Furthermore, H_2SO_4 increases the sulfur (S) content to a level that prohibits extensive use of acidified slurry in anaerobic digestion biogas plants due to the inhibition of the biogas process (Moset et al., 2012). Additionally, the handling of strong concentrated acids is hazardous and problems may arise related to its utilisation, such as equipment corrosion and foam formation (Fangueiro et al., 2015).

Therefore, an alternative to the traditional acidification of slurry using H_2SO_4 is required in order to facilitate proper slurry treatment and reuse of the slurry following acidification. Organic acids have previously

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been studied in relation to reducing NH_3 emissions (Regueiro et al., 2016; Clemens et al., 2002), but the associated costs are very high and therefore a more cost-effective sustainable treatment is required.

Bio-acidification of animal slurry is a microbially mediated process that occurs under anaerobic conditions whereby the slurry is acidified into organic acids produced by native or added microorganisms (Nykänen et al., 2010). This process can be induced by adding easily degradable organic substrates that are metabolised in a fermentation process by the microorganisms present in the slurry (Clemens et al., 2002; McCrory and Hobbs, 2001). Lactic acid is one of the main organic acids produced and is capable of decreasing the slurry pH below 4.5 (Bastami et al., 2016) due to its low pKa value of 3.8. To ensure that lactic acid is produced rather than other organic acids such as acetic acid, homofermentative bacteria should predominate over hetero-fermenters (McDonald., 1982). Adjusting the slurry pH by adding labile carbon sources or acid should decrease the concentration of bacteria producing undesired metabolites (Nykänen et al., 2010) and stimulate lactic acid-producing bacteria. Therefore, H_2SO_4 can be used to pre-acidify the slurry and study the possible inhibition effects, which may prevent bacterial activity other than that of lactic acid fermenters, and whether the initial fall in pH with acid reduces the amount of glucose required to induce the fermentation process. Such acidification of the medium typically prevents other undesired microbial activity, such as acetogens and methanogens, and thus avoids formation of CH_4.

Similar to traditional acidification, bio-acidification has the potential to reduce NH_3 and CH_4 emissions while aiming at nutrient recovery with bio-acidified products for use as fertilisers or for green energy recovery by anaerobic digestion for biogas production. To date, few studies have assessed NH_3 and GHG emission reductions through the addition of agricultural waste. Bastami et al. (2016) demonstrated 70 % and 31 % reductions in CH_4 emissions with a 7 % and 7.7 % inclusion of brewers' spent grain and milk, respectively. The addition of 3–33 % sugar beet residues resulted in an NH_3 volatilisation reduction of between 5 % and 26 % (Clemens et al., 2002), while 50 % inclusion of cheese whey reduced NH_3 volatilisation by 68 %, but equal GHG emissions were observed relative to the control (Prado et al., 2020). A recent study showed a 67 % reduction in NH_3 emissions from cattle slurry amended with 5 % sugar beet molasses, and CH_4 reductions ranging from 15 % to 70 % with a larger inclusion of the substrate (Kavanagh et al., 2021). It is crucial to establish the correct substrate dosage to induce the fermentation process in order to reduce NH_3 but also CH_4 and CO_2 emissions, which might otherwise increase due to the input of labile carbon (C) sources. The ideal substrate to add to the slurry would be a waste or residue biomass with little economic value and a sufficient content of easily degradable carbohydrate to stimulate slurry fermentation. One such residue is brown juice, which is the liquid residue obtained during the extraction of protein from grass and other types of fresh green plant biomass (Santamaría-Fernández and Lübeck, 2020). Brown juice typically has a relatively high sugar content and low pH, but is currently of relatively low economic value; it is mostly considered suitable as a co-substrate in anaerobic digestion plants for biogas production. Therefore, encouraging a better use of resources, such as utilising agricultural waste to reduce gaseous emissions, while gaining value from reused slurries that limits dependency on finite resources such as mineral fertilisers, would contribute to a more sustainable agricultural practice and a bio-based circular economy.

The objective of this study was to assess the efficiency of the slurry bio-acidification treatment by adding different amounts of substrate (glucose, brown juice, sulfuric acid or a mix of these) in order to initiate the fermentation process. The stability of the treatments was evaluated by quantifying the most important pools and fluxes of carbon (glucose, organic acids, and CH_4 and CO_2 emissions) and monitoring them over time in order to understand the metabolic mechanisms involved during the treatment.

The hypotheses of this study were that:

i) the greater the concentration of sugars added, the larger the quantity of acids produced during bio-acidification and the lower the pH
ii) if additional acid is added, lesser a lower concentration of sugar is needed to establish a stable low pH
iii) reaching a pH of 4.2 will inhibit the production of weaker volatile fatty acids (VFAs), such as butyric, propanoic and acetic acid, rather than of lactic acid.

2. Material and methods

2.1. Feedstocks

Fresh pig slurry with an initial pH value of 8.3, a total solids (TS) content of 2.5 % and a volatile solids (VS) content of 1.4 % was collected from the storage tank of a farm in Funen, Denmark in September 2019. The farm is a slaughter pig production facility where pigs are grown from the start weight of 30 kg until they reach 110 kg.

Brown juice was produced from protein-rich green juice obtained by screw-pressing fresh biomass from a clover and grass sward using an Angelia 8500S juicer. Two types of brown juice were used that were obtained by: 1) heat treatment of the green juice at 85 °C, followed by centrifugation to precipitate the coagulated proteins, and 2) lactic acid fermentation where an overnight inoculum of *Lactobacillus salivarius* (Santamaría-Fernández et al., 2017) was added to the green juice and incubated at 38 °C for 6–8 h until the pH was 3.8, followed by centrifugation to precipitate the proteins. Brown juice from coagulation had an initial pH of 5.5, TS of 6.7 % and VS of 4.6 %, with concentrations of 25 g glucose / L brown juice and 0 g lactic acid/L brown juice. Brown juice from fermentation had a lower initial pH than brown juice from coagulation, with a pH value of 3.8, TS of 6.7 %, VS of 4.8 % and 12 g of glucose/L brown juice and 28 g lactic acid / L brown juice, which was the main component responsible for the low pH.

2.2. Experimental set-up

Samples of 300 g fresh slurry were either unacidified or preacidified with sulfuric acid to initial values of pH 5.5 and pH 5 and combined with different concentrations of glucose (0 %, 2 % and 4 %) and the two types of brown juice at different levels (20 % and 50 %, w.w.). The amount of each additive was selected through a short-term preliminary screening experiment performed in the laboratory, during which the impact of the additive on pH evolution was monitored for 20 days (treatments and results in supplementary material). A total of 16 different treatments were applied (Table 1) with three replicates. The different combinations of substrates selected in the treatments were comparable by their glucose concentrations (Table 2) with or without brown juice addition and assessed with the effect of preacidifying with H_2SO_4 on the reduction of glucose needed to induce the fermentation process.

The additives were gradually added to the slurries with continuous stirring, and the pH after each addition was measured using a combined electrode (PHM 210 Meter lab pH meter, Radiometer Medical ApS, Brønshøj, Denmark).

Each slurry sample was divided into three 100 g subsamples that were placed in 120 mL plastic containers inside airtight glass vessels of 0.75 L capacity. The vessels were fitted with a rubber septum in the lid for gas sampling and kept under undisturbed and anaerobic conditions, which were ensured by flushing the headspace of the vessel with N_2 after each sampling. Treatments and control slurry samples (unamended) were incubated for 98 days at ambient room temperature (approx. 20 °C).

2.3. Chemical analysis

The TS content was determined by drying 10 g of fresh material at 105 °C for 24 h to constant weight, and the VS content was determined

Table 1
Treatments performed with substrates and acid dosages.

Treatments	Slurry added (g)	Glucose added (g)	BJcoag added (g)	BJferm added (g)	H_2SO_4 added (mL)	Initial pH
RS–noC	100	–	–	–	–	8.3
RS–glu2	98	2	–	–	–	8.2
RS–glu4	96	4	–	–	–	8
RS–glu2–coag50	48	2	50	–	–	7
RS–glu2–ferm50	48	2	–	50	–	5.7
RS–glu4–coag20	76	4	20	–	–	7.7
RS–glu4–ferm20	76	4	–	20	–	6.8
acid5.5–noC	99.4	–	–	–	0.58	5.5
acid5.5–glu2	97.4	2	–	–	0.57	5.5
acid5.5–glu4	95.4	4	–	–	0.56	5.5
acid5.5–glu2–coag20	77.5	2	20	–	0.46	5.5
acid5.5–glu2–ferm20	77.5	2	–	20	0.46	5.1
acid5–noC	99.3	–	–	–	0.66	5
acid5–glu2	97.4	2	–	–	0.65	5
acid5–glu2–coag20	77.5	2	20	–	0.52	5
acid5–glu2–ferm20	77.5	2	–	20	0.52	4.6

BJcoag: brown juice from coagulation; BJferm: brown juice from fermentation.

Table 2
Initial treatment characteristics.

Treatments	Initial pH	TS (g kg mixture^{-1})	Glucose (g L^{-}mixture^{-1})	Lactic acid (g L mixture^{-1})	Total VFAs (g L mixture^{-1})
RS–noC	8.3	25.3 ± 0.4^{j}	0.0 ± 0.0^{j}	0.0 ± 0.0^{d}	8.3 ± 0.5^{a}
RS–glu2	8.2	41.9 ± 0.9^{i}	20.4 ± 0.2^{i}	0.0 ± 0.0^{d}	6.8 ± 0.0^{bc}
RS–glu4	8	57.2 ± 0.9^{f}	40.6 ± 0.1^{c}	0.0 ± 0.0^{d}	4.4 ± 1.0^{f}
RS–glu2–coag50	7	59.4 ± 0.5^{def}	32.5 ± 0.0^{d}	0.0 ± 0.0^{d}	3.7 ± 0.1^{f}
RS–glu2–ferm50	5.7	63.3 ± 0.8^{cde}	26.5 ± 0.2^{e}	13.8 ± 0.1^{a}	4.0 ± 0.0^{ef}
RS–glu4–coag20	7.7	66.5 ± 1.0^{bc}	44.4 ± 0.4^{a}	0.0 ± 0.0^{d}	5.4 ± 0.0^{cde}
RS–glu4–ferm20	6.8	64.1 ± 0.5^{bcd}	42.7 ± 0.1^{b}	5.5 ± 0.0^{b}	5.6 ± 0.0^{bcd}
acid5.5–noC	5.5	51.6 ± 0.3^{gh}	0.0 ± 0.0^{j}	0.0 ± 0.0^{d}	7.0 ± 0.1^{ab}
acid5.5–glu2	5.5	67.1 ± 1.1^{bc}	21.4 ± 0.4^{hi}	0.0 ± 0.0^{d}	7.1 ± 0.3^{ab}
acid5.5–glu4	5.5	81.7 ± 0.4^{a}	42.0 ± 0.8^{bc}	0.0 ± 0.0^{d}	6.5 ± 0.1^{bc}
acid5.5–glu2–coag20	5.5	69.3 ± 1.5^{b}	24.3 ± 0.2^{f}	0.0 ± 0.0^{d}	4.9 ± 0.0^{def}
acid5.5–glu2–ferm20	5.1	67.5 ± 2.7^{bc}	23.5 ± 0.5^{fg}	5.5 ± 0.1^{b}	4.9 ± 0.0^{def}
acid5–noC	5	37.3 ± 1.0^{i}	0.0 ± 0.0^{j}	0.0 ± 0.0^{d}	4.6 ± 0.1^{def}
acid5–glu2	5	50.5 ± 0.3^{h}	21.1 ± 0.1^{hi}	0.0 ± 0.0^{d}	4.4 ± 0.1^{def}
acid5–glu2–coag20	5	58.5 ± 0.7^{ef}	23.7 ± 0.2^{fg}	0.0 ± 0.0^{d}	3.7 ± 0.0^{f}
acid5–glu2–ferm20	4.6	56.5 ± 0.6^{fg}	22.2 ± 0.5^{gh}	5.3 ± 0.0^{c}	3.5 ± 0.3^{f}

TS: total solids; total VFAs is the sum of acetic, propionic, butyric, isobutyric, valeric, isovaleric and hexanoic acid.
All values are presented (on a wet weight basis) as mean values of three replicates with standard errors. Means followed by different letters within the same column are significantly different from each other ($p < 0.05$).
For treatment abbreviations, see Table 1.

by loss on ignition after calcination at 550 °C for 4 h.

The evolution of pH was followed by measurement of the pH every week. The measurements were performed at the bottom of the vessel, avoiding the slurry surface. Before measuring the pH, each jar was opened for 10 min for aeration to liberate other gaseous emissions, such as CO_2, that increase slurry pH and could interfere with slurry pH measurements (Hafner et al., 2012).

Every week before opening the glass jars, gas samples for CO_2 and CH_4 gaseous emissions measurements were taken from the glass jar headspace and stored in pre-evacuated vials and analysed by gas chromatography (GC) using a Bruker 450-SC (Germany) equipped with a TDC and ECD, and the separation was achieved by using a packed column filled with Porapak QS. The oven temperature was 50 °C and the carrier gas was argon. Results were calibrated against certified gas standards (Air Products, Waltham-on-Thames, UK).

Production of CO_2 and CH_4 was calculated from the gas concentrations, taking into account the seven-day sampling time difference, the headspace volume variation of the jar and the volume variation of the slurry.

Every two weeks, a 5 g sample was taken from the jar to analyse the glucose consumed and lactic acid produced, as well as by-products such as volatile fatty acids. After every jar opening at sampling time, the vessels were flushed with N_2 to release O_2 from the jars' headspace and maintain anaerobic conditions.

Sugars and lactic acid were determined by HPLC on a Dionex Ultimate 3000-LC system with an Aminex® HPX-87H column coupled to a refractive index detector. H_2SO_4 (4 mmol L $^{-1}$) was used as the mobile phase, with a flow rate of 0.6 mL min^{-1} at 60 °C. The concentration of acetic, propionic, butyric, isobutyric, valeric and isovaleric acids (and the sum of these calculated as total volatile fatty acids (TVFA)) were determined using a gas chromatograph (PerkinElmer, Clarus 400), equipped with an Agilent HPFFAP capillary column of 30 m length and 0.53 mm i.d. followed by a flame ionisation detector (FID). The carrier gas (used to evaporate the fatty acids dissolved in the slurry sample) was nitrogen (13 mL min^{-1}), and the respective temperatures of the detector and injector were 230 °C and 240 °C.

2.4. Statistics

In order to evaluate the effect of the performed treatments on pH evolution, glucose consumption, lactic acid production and total volatile fatty acids production, a mixed analysis of variance (ANOVA) with repeated measures was used, with measurement day as the repeated variable. When the ANOVA with repeated measures revealed a significant interaction of treatment * day, the treatment effect was conducted at each sampling time (0, 14, 28, 56, and 98 d) using one-way ANOVA. The significance of the differences was compared two by two with a Tukey test at the $p < 0.05$ level. The normality and homogeneity of

variances were checked by the Shapiro-Wilk and Levene test respectively. The IBM SPSS Statistics v.27 software package for Windows was used for the statistical analyses (IBM Inc., Chicago, IL).

3. Results and discussion

3.1. Effect of treatments on pH

The effect of substrate addition on pH evolution, glucose consumption and lactic acid production during the 98 days storage period is shown in Fig. 1a to i. The initial pH decrease promoted by the addition of substrates was shown to have an impact on facilitating the lactic acid fermentation process. Lower initial pH values together with easily degradable carbohydrate supplementation helped optimal conditions to be reached for acidogens to grow, and thus inhibited acetogen and methanogen metabolic activity. During acidogenesis, the organic matter present in the slurry is fermented into lactic acid, VFAs and alcohols by the microbes present in the slurry, with a consequent further decrease in pH depending on the organic acid formed and its corresponding pKa. Two types of lactic acid fermentation can take place depending on the bacteria present in the slurry: homolactic fermentation, where only lactic acid is produced, and heterolactic fermentation, where lactic acid is produced along with acetic acid, citric acid and ethanol (Rogers et al., 2013; McDonald, 1982). If mainly lactic acid (pKa 3.8) is produced, the pH will decrease markedly, lactic acid bacteria (LAB) will be the predominant bacterial community present, and other acidogens will not be able to compete for the substrate. By reaching pH 5 or lower, production of VFAs such as valeric, propanoic, butyric and acetic acid with pKa values of 5.01, 4.88, 4.82 and 4.76 respectively, may be inhibited, as lower pH values promote ideal conditions for acidogens to grow, specifically LAB, and inhibit microbial activity from other fermenting bacteria.

Raw pig slurry (RS) had an initial pH of 8.3, which increased throughout the storage period until it reached a pH of 8.9 at the end of storage (Fig. 1a). The addition of substrates to non-preacidified slurries led to the pH decreasing from its initial value, both initially but also over time. The decrease in initial pH was more significant at higher initial glucose concentrations, thus the addition of 2 % glucose did not have a significant impact on initial pH, whereas 4 % glucose addition decreased the initial pH to 8, and lower initial pH values were reached when glucose was combined with brown juice from fermentation. The lowest initial pH of 5.7 in non-preacidified slurries was reached when adding 2 % glucose in combination with 50 % brown juice from fermentation (RS–glu2–ferm50), followed by RS–glu4–ferm20, where the initial pH dropped to 6.8 (Fig. 1a). The significantly ($P < 0.05$) large decrease in initial pH in RS–glu2–ferm50 was mostly due to the acidic nature of the brown juice substrate from fermentation (Table 2), which is a result of bacterial sugar fermentation containing lactic acid and an initial pH of 3.8. Kavanagh et al. (2021) obtained similar results of immediate pH reduction in cattle slurry when using acidic substrates such as grass and

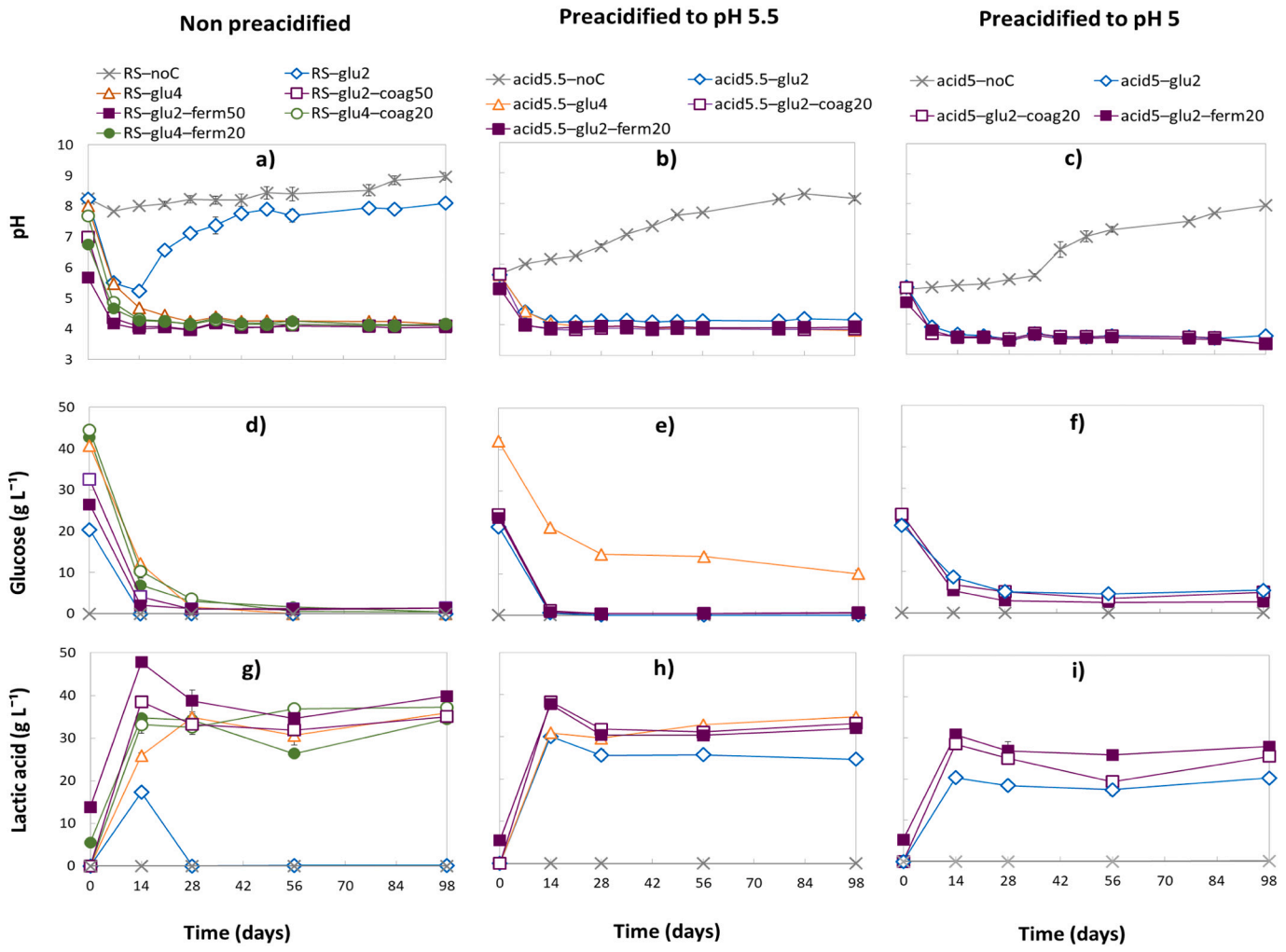

Fig. 1. Treatment effect on pH (a-c), glucose consumption (d-e) and lactic acid production (g-i) over time. Error bars represent the standard error of the mean ($n = 3$). For abbreviations of treatments, see Table 1.

maize silage effluents at 15 % inclusions.

The extra addition of brown juice from fermentation to preacidified slurries had the same effect as in non-preacidified slurries by further decreasing the initial pH, with a significant ($P < 0.05$) decrease in initial pH to 5.2 in acid5.5–glu2–ferm20 (Fig. 1b) and to 4.7 in acid5-–glu2–ferm20 (Fig. 1c).

Subsequently, the pH decreased efficiently with all treatments until day 14, except in the control raw slurry (RS-noC), in RS–glu2, and in preacidified slurries with no C source (acid5.5-noC and acid5-noC) (Fig. 1a-c). The decrease in pH was kept stable throughout the storage, with a more pronounced pH drop at higher C or acid dosage. However, after the decrease of pH in RS–glu2 to pH 5.3 at day 14, pH subsequently increased rapidly until the end of the storage, reaching the initial RS pH of 8.3 (Fig. 1a). The pH in the acid5.5–noC treatment without added C increased continuously from pH 5.5 at the beginning of storage to pH 8.3 (Fig. 1b), similar to the initial pH of RS, and even the acid5–noC treatment increased slowly to pH 5.5 at day 35, but then increased markedly until it reached pH 8 at the end (Fig. 1c).

The buffering capacity of the slurry is very complex and is mainly influenced by the contents of ammoniacal N, carbonates, phosphates and volatile fatty acids (Sommer and Husted, 1995), which directly affect the slurry pH and its stability during storage. The increase in pH observed in preacidifed slurries to pH 5.5 and pH 5 without substrate addition (acid5.5-noC and acid5-noC) from the beginning of the storage (Fig. 1b-c) can be explained by degradation of dissociated organic acids. The same tendency has previously been observed by Regueiro et al. (2016), Petersen et al. (2012) and Eriksen et al. (2008), who attribute this pH increase to microbial oxidation in the slurry where bacteria take up acids in dissociated forms with the release of hydroxyl ions and consequent increase in pH. This microbial decomposition was also confirmed with the release of CO_2 observed in these treatments throughout the storage period (Fig. 2e-f).

Non-preacidified slurries showed the significantly ($P < 0.05$) lowest pH of 3.9 at day 28 when 2 % glucose was added in combination with 50 % brown juice (RS-glu2-coag50 and RS-glu2-ferm50) (Fig. 1a). The pH slightly increased at day 35, after which no more significant differences were observed when compared with the rest of the non-preacidifed treatments (except RS– glu2). Similar values in pH reduction after seven days were obtained by Bastami et al. (2016) when 10 % glucose was added to cattle slurry. This suggests that the combination of 2 % glucose with 50 % brown juice from fermentation in the present study had similar effects on the pH decrease, partly due to the extra sugar content in brown juice, but mostly due to its acidic nature provided by the lactic acid content (Table 2) and probably the remaining LAB from the fermentation process.

Preacidified slurries to pH 5.5 showed a significantly ($P < 0.05$) lower pH of 3.9 at day 14 when 2 % glucose was combined with 20 % of both types of brown juice (Fig. 1b). However, from day 28 the pH value did not show any significant difference from the 4 % glucose treatment (acid5.5-glu4), with pH values of 3.9 that were maintained until the end of the storage. Acid5.5–glu2 and acid5.5–glu4 did not show significant pH differences until day 14 (Fig. 1b), but were significantly ($P < 0.05$) different from day 14, with acid5.5–glu2 having a significant ($P < 0.05$) pH increase. Preacidified slurries to pH 5 did not show any significant difference between treatments throughout the storage period, except for acid5–noC (Fig. 1c).

3.2. *Effect of treatments on glucose consumption and lactic acid production*

Lactic acid production occurred in all treatments throughout the storage period while some glucose was present, but no lactic acid was produced in treatments where no C substrate was added (RS-noC, acid5.5-noC and acid5-noC, Fig. 1. g-i). The significant ($P < 0.05$) increase in lactic acid concentration compared with the treatments where no substrate was added would therefore appear to explain the bioacidification and pH decrease promoted by substrate addition. The increase in lactic acid concentration was shown to be correlated with the decrease in pH observed during storage in all treatments, except in treatments where no substrate was added and in RS-glu2 (Fig. 3), where lactic acid was only produced until day 14 (Fig. 1g). This confirmed that high lactic acid concentrations led to a low pH.

Lactic acid concentration reached the highest value in most of the treatments at day 14, then decreased from day 14 to 28 in treatments where glucose was almost completely consumed, but slightly increased afterwards until the end of the storage (Fig. 1 g-i). Lactic acid was

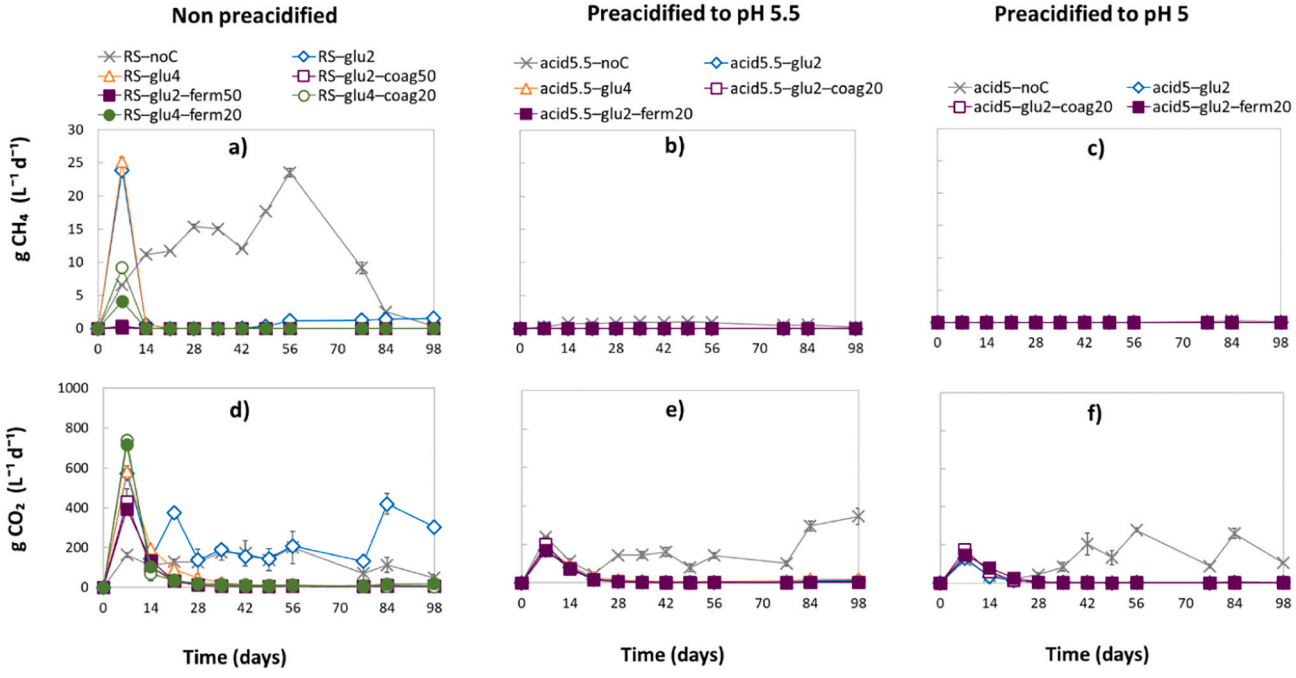

Fig. 2. Emissions of CH_4 and CO_2 during storage of treatments. Error bars represent the standard error of the mean (n = 3). For abbreviations of treatments, see Table 1.

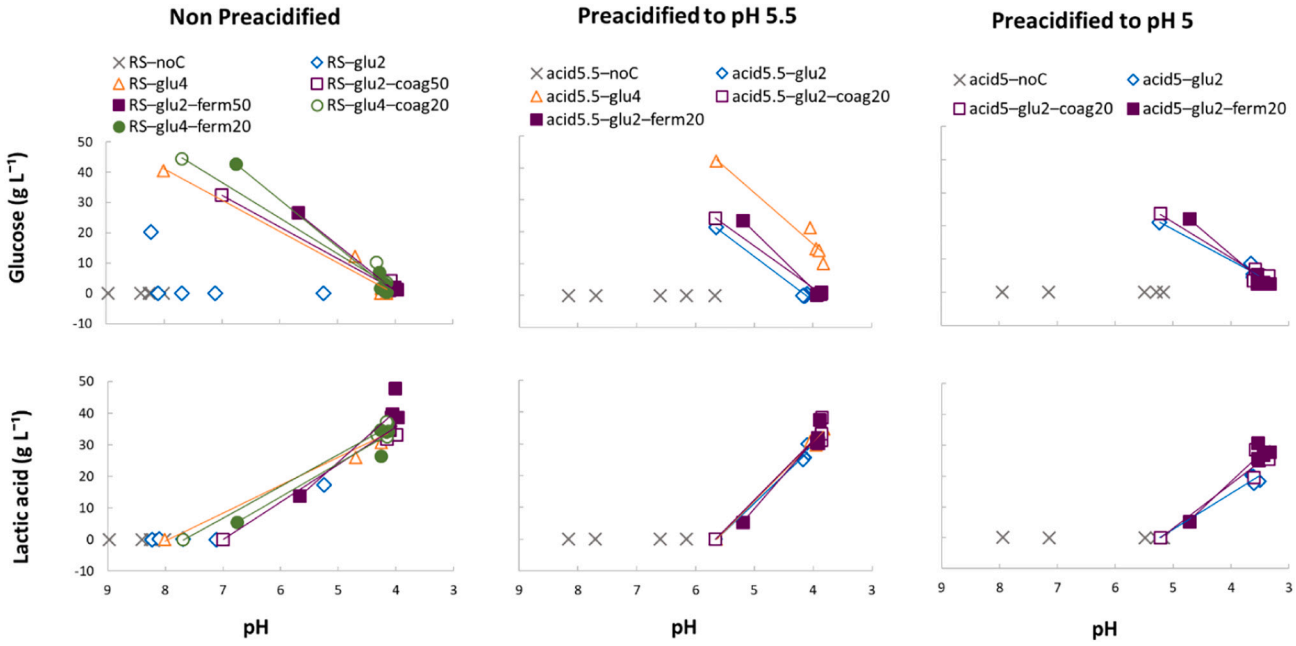

Fig. 3. Correlations between pH and glucose concentration (top) and lactic acid concentration (bottom) throughout the storage period. For treatment abbreviations, see Table 1.

produced in RS-glu2 until glucose was completely consumed at day 14 (Fig. 1d), when lactic acid concentration started to decrease until negligible values were reached at day 28 (Fig. 1g). The low pH observed in RS-glu2 while the concentration of lactic acid was high rose drastically from day 14 (Fig. 1a), suggesting that lactic acid conversion into other less acidic compounds was mainly responsible for this pH increase (Fig. 4). The increase in pH after 14 days together with the disappearance of lactic acid suggests that methanogenic activity (consumption of VFAs) or a secondary fermentation (consumption of lactic acid into other weaker VFAs) started to occur. From day 14 a significant increase in CO_2 production was observed in RS-glu2 (Fig. 2d) relative to the other treatments, which together with the increase in valeric acid at day 28 (Fig. 4) suggest secondary fermentation to be mainly responsible for this pH increase. The same tendency was observed in preacidified slurries where no substrate was added (acid5.5-noC and acid5-noC, Fig. 1h-i). After day 42, however, small increases in CH_4 production were observed (Fig. 2b-c), which together with the reduction in VFAs (Fig. 4) confirmed that methanogenic activity took place.

The significantly ($P < 0.05$) highest lactic acid concentration from all treatments was observed in RS-glu2-ferm50 at day 14 (Fig. 1g), which showed the highest pH correlation ($r = 0.99$) with the glucose being consumed (Fig. 3). Even though RS-glu2-ferm50 had a significantly ($P < 0.05$) lower initial glucose concentration (Fig. 1d) than all of the non-preacidified treatments (except RS-glu2), the high lactic acid concentration reached at day 14 suggested that the initial lactic acid content (Table 2) supplied by the use of brown juice from fermentation together with extra LAB present may have contributed to the greater production of lactic acid. The high concentration in lactic acid, along with the avoidance of formation of other less acidic organic acids (Fig. 4), indicates that lactic acid content was mainly responsible for the bioacidification, which is in accordance with the lowest pH reached (Fig. 1a), and the initial LAB content in the fermented brown juice may have helped trigger the bioacidification process. Despite glucose in RS-glu2-ferm50 being almost completely consumed at day 28 (Fig. 1d), increases in glucose were observed from day 56 until the end of the storage, suggesting that some hydrolysis of hemicelluloses in the slurry, for example, may have occurred. The low pH maintained with this treatment throughout the experiment suggested that lactic acid bacteria were very well established and no other microbial activity was present since no CH_4 or CO_2 was produced during the storage of this treatment (Fig. 2). When the substrate is not limiting, LAB dominate the fermentation producing lactic acid, which decreases pH and suppresses LAB growth, resulting in a stable process (Rooke and Hatfield, 2003).

The trends in glucose degradation were similar in RS-glu2-coag50 and RS-glu2-ferm50, however lactic acid concentration was significantly ($P < 0.05$) lower in RS-glu2-coag50 (Fig. 1g) as no initial lactic acid was present. A similar lactic acid content was observed at day 14 in RS-glu2-coag50 (Fig. 1g) to that in acid5.5-glu2-ferm20 and acid5.5-glu2-coag20 (Fig. 1h), suggesting that preacidifying to pH 5.5 with H_2SO_4 had similar effects in the amount of lactic acid produced to the addition of extra 30 % brown juice from coagulation to non-preacidified treatments.

Non-preacidified treatments with higher initial glucose concentrations (RS-glu4-coag20, RS-glu4-ferm20 and RS-glu4, Table 2) were shown to have an effect on the lactic acid produced at a later stage and formation of by-products. These three treatments reached their highest lactic acid production at day 28 compared with the rest of the treatments reaching it at day 14 (Fig. 1g). Even though lactic acid concentrations increased at a later stage (day 56) in these treatments, they reached lactic acid concentrations similar to RS-glu2-ferm50 at the end of the storage. However, treatments with a higher initial glucose concentration showed a lower correlation ($r = 0.97$) between glucose and pH (Fig. 3) and a higher concentration of VFAs produced (Fig. 4) than RS-glu2-ferm50. The high initial glucose concentration in RS-glu4 was depleted at day 56, while some remaining glucose was still recorded at the end of the storage in RS-glu2-ferm50, which had a significantly ($P < 0.05$) lower initial glucose concentration (Table 2).

Preacidification of slurries, both to pH 5.5 and pH 5, had a significant ($p < 0.05$) effect on the glucose consumption rate. Acidification with sulfuric acid may inhibit microbial acidogenesis (Hjorth et al., 2015b), and this was corroborated by a comparison of glucose consumption trends in treatments with the same initial glucose concentrations with/without previous acidification. The initial glucose concentration in RS-glu4 without preacidification was almost completely consumed at day 28 and completely consumed at day 56 (Fig. 1d), while the same initial glucose concentration in the same preacidified treatment (acid5.5-glu4) was not completely consumed at the end of the storage (Fig. 1e). The same trend occurred when comparing acid5.5-glu2 and acid5-glu2,

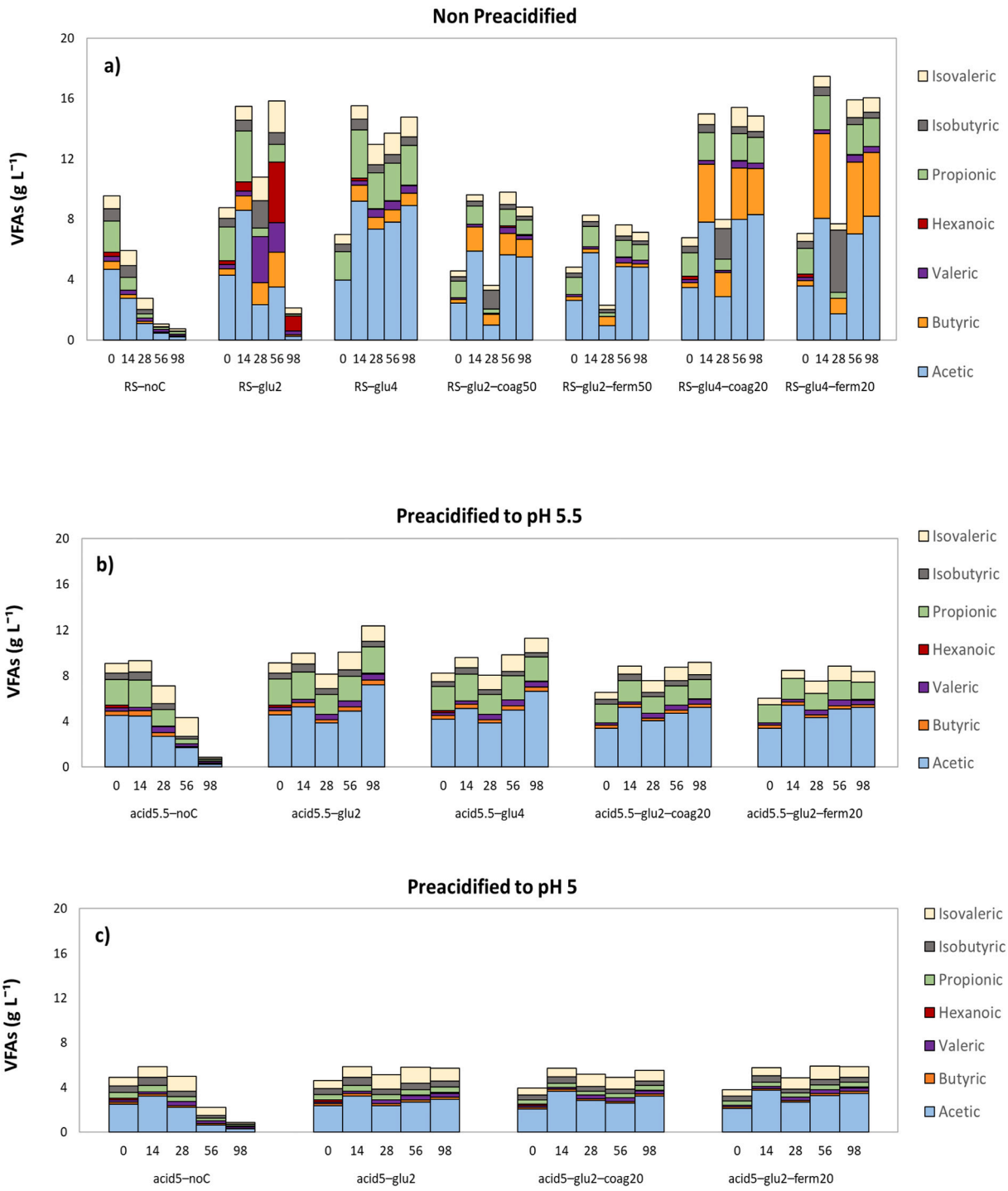

Fig. 4. Treatments effect on volatile fatty acids concentration during storage. For abbreviations of treatments, see Table 1.

where the same initial glucose concentration (Table 2) was finished in acid5.5-glu2 at day 14 (Fig. 1e), while glucose still remained at the end of the storage in acid5-glu2 (Fig. 1f). This inhibition had an effect on the lactic acid produced, as lactic acid decreased from day 14 to 56 in all treatments acidified to pH 5 while there was still residual glucose. Glucose increased at the end of the storage, probably due to hydrolysis occurring at these very low pH values (as also happened when no C was added), which suggests hydrolysis of a more complex carbohydrate content in the slurry.

3.3. *Effect of treatments on VFAs and ethanol production and emissions of CO_2 and CH_4*

Raw pig slurry (RS) had a high initial concentration of total VFAs (9.5 g L^{-1}), as seen in Fig. 4, mostly due to acetic and propionic acid. These VFAs were consumed from the outset and throughout the storage period to trace levels at the end of the storage. This shows methanogenic activity was happening from the start of the storage, which was confirmed by the production of CO_2 and especially CH_4, increasing from day 0 until day 56 and slightly decreasing afterwards (Fig. 2) once the VFAs were consumed (Fig. 4). Preacidified slurries to pH 5.5 and pH 5

without substrate addition (acid5.5-noC and acid5-noC) showed the same trends in terms of VFA consumption, where a significant VFA decrease was observed from day 56 once the pH was above 7 and methanogens had ideal conditions in which to grow.

Volatile fatty acids in non-preacidifed slurries increased during the first 14 days of storage when substrates were added, especially acetic and propionic acid (Fig. 4). Butyric acid increased significantly in RS-glu4-coag20 and RS-glu4-ferm20 at day 14, suggesting that microorganisms other than LAB, such as clostridia, were not suppressed and might also be growing. Rooke and Hatfield (2003) stated that if the substrate is limited or the fermentation rate is slow, these enterobacteria may be present and transform lactic acid into butyric acid. As previously explained, these treatments had the highest initial glucose concentration, however the glucose consumption rate was lower than in treatments with a lower initial glucose content, which is a possible explanation for the appearance of butyric acid.

When adding 2 % glucose to pig slurry (RS-glu2), VFAs significantly increased during the first 14 days of storage, especially acetic and butyric acid, probably due to acidogenesis as CO_2 was released during this period (Fig. 2). After 14 days of storage, glucose was finished in RS-glu2 and, along with the degradation of lactic acid, degradation of VFAs started with the consequent pH increase. Acetic acid was reduced from day 14 and butyric and valeric acids increased as by-products of the secondary fermentation of lactic acid. Methanogenic activity took place from day 42 with the release of CH_4 (Fig. 2) and hexanoic acid increased significantly, to be consumed afterwards together with the rest of the VFAs. Therefore an extra addition of substrate would be required in RS-glu2 to avoid lactic acid conversion into weaker VFAs.

When 4 % glucose was added to pig slurry (RS-glu4), VFAs also greatly increased in the first 14 days. Glucose still remained, therefore lactic acid did not decrease between day 14 and 28, but did decrease from day 28 once the glucose was finished. Acetic acid was the predominant VFA in RS-glu4 and was significantly ($P < 0.05$) higher than in RS-glu2, and may be mainly responsible for keeping the pH below 4.5 until the end of the storage.

Organic acids, such as butyric and propionic acid, significantly increased in RS-glu2, RS-glu4, RS-glu4-coag20 and RS-glu4-ferm20 at day 14 (Fig. 4). These treatments had initial pH values higher than 5.5, and at day 14 did not reach pH values below 4.2, suggesting that acidogens other than LAB were present to produce these VFAs.

However, the RS-glu2-ferm50 treatment had an initial pH of 5.7 due to the lactic acid provided by the addition of brown juice from fermentation (Table 2), and this treatment reached a pH of 4.2 at day 14 (Fig. 1a), which may create ideal conditions for LAB to grow. Therefore, by-products from the activity of other acidogens, such as propionic and butyric acid, were not produced (Fig. 4). This treatment, RS-glu2-ferm50, showed the lowest pH values throughout the storage period, with less weak VFAs produced and the highest lactic acid concentration, with some glucose remaining at the end of the storage, suggesting that lactic acid and LAB present in this treatment had a positive impact on maintaining a stable fermentation process, even though the initial glucose concentration was lower than in other treatments since microbial activity other than LAB may have been inhibited.

Acidification to pH 5.5 and pH 5 reduced the total VFA content of raw slurry, however the VFA content increased (mostly propionic acid) when acidification to pH 5.5 was combined with substrate addition. This was probably due to hydrolysis (e.g. hemicelluloses in the slurry) promoted by acidification and subsequent fermentation into VFAs. Acidification to pH 5, however, decreased the total VFA content, probably due to substrate or bacterial inhibition. Even though the initial VFA content was reduced to pH 5.5 in preacidifed slurries, the increase from day 0 to 14 was significantly ($P < 0.05$) lower compared with non-acidified slurries. This may be due to the bacterial activation promoted by acidification, as the lactic acid produced during this period was greater in slurries preacidified to pH 5.5 than in non-preacidified slurries with the same substrate dosage, although the glucose consumption rate was lower. In general, preacidification to pH 5.5 did not demonstrate any additional advantage compared with non-preacidified slurries, and more propionic acid was observed in slurries preacidified to pH 5.5. The remaining glucose at the end of the storage and lower lactic acid concentration than in the same non-preacidified treatments suggest that acidification slows down acidogenesis and therefore lactic acid production, with the consequent production of weaker VFAs such as propionic acid.

Few studies have monitored VFAs during organic treatment of slurries with acidification purposes. Bastami et al. (2016) studied VFAs during the storage of slurry acidified with brewing sugar to reduce CH_4 emissions and found inconsistent VFA production, and therefore the mechanism for self-acidification was not clear. However, some studies have shown how co-ensiling of cattle manure with glucose addition is possible depending on the glucose concentration added and manure composition (Franco et al., 2018). They found that with a 10 % glucose addition, lactic acid fermentation persisted after four months of storage. However, 4 % glucose was consumed after a month of storage and consequently a degradation of lactic acid into VFAs, such as butyric acid, was observed.

One of the main concerns when adding easily degradable sources of C to slurries is the increase in C, especially through CH_4 emissions. Methane fluxes in non-preacidified treatments were in the range of previous studies by Kavanagh et al. (2021), Prado et al. (2020) and Bastami et al. (2016). The addition of substrates promoted an initial peak in CH_4 release during the first week in all treatments, except for RS-glu2-ferm50 (Fig. 2). However this peak was only significantly ($P < 0.05$) higher in RS-glu and RS-glu4, with values in the same range as those reported by Bastami et al. (2016). Methane emissions were negligible after day 7 in all treatments, except in RS-glu2 where some methanogenic activity was observed from day 42 (Fig. 2a). The same trend was observed in CO_2 emissions, with a significantly ($P < 0.05$) higher peak during the first week in treatments with a higher initial glucose concentration (RS-glu4-coag20, RS-glu4-ferm20 and RS-glu4, Table 2). This trend was also observed by Prado et al. (2020) and was attributed to the increased microbial activity within the period of pH decrease. Carbon dioxide emissions remained at negligible values in all treatments from day 21 and the remaining storage period, except in RS-noC and RS-glu2, where CO_2 emissions were significantly higher ($P < 0.05$) throughout the storage period. Emissions of CO_2 were also observed in preacidified slurries where no substrate was added (acid5.5-no C and acid5-noC, Fig. 2e-f).

Ethanol was not initially present in any of the treatments and was produced in all of them from the beginning of the storage (Fig. 5a-c), except when no C substrate was added (RS-noC, acid5-noC and acid5.5-noC) in RS-glu2 and RS-glu4-ferm20 (Fig. 5a). Ethanol production followed the same trend as lactic acid production during the first two weeks of storage (Fig. 1g-i), with significantly ($P < 0.05$) higher ethanol concentrations in treatments where the lactic acid concentration was higher, except for RS-glu4-ferm20 for which there was no explanation for the absence of ethanol. Ethanol production suggested that heterolactic LAB was probably present or a combination of both types of LAB, homolactic and heterolactic, was present and active. The ethanol concentration decreased afterwards until the end of the storage, which was probably due to a predominant homolactic fermentation taking over the initial heterolactic fermentation (Schleifer and Ludwig, 1995). The same trend in ethanol production was observed in preacidified slurries, with acid5.5-glu2-ferm50 showing significantly ($P < 0.05$) higher values at day 14 (Fig. 5b-c). The switch in fermentations after two weeks was confirmed with the correlation between LA and the ethanol produced (Fig. 6).

Citric acid was initially present in all the treatments, with significantly ($P < 0.05$) higher concentrations in treatments with a higher percentage of added brown juice (Fig. 5d-f). The citric acid decreased during storage until it was completely consumed at day 28, confirming homolactic fermentation taking over the initial heterolactic

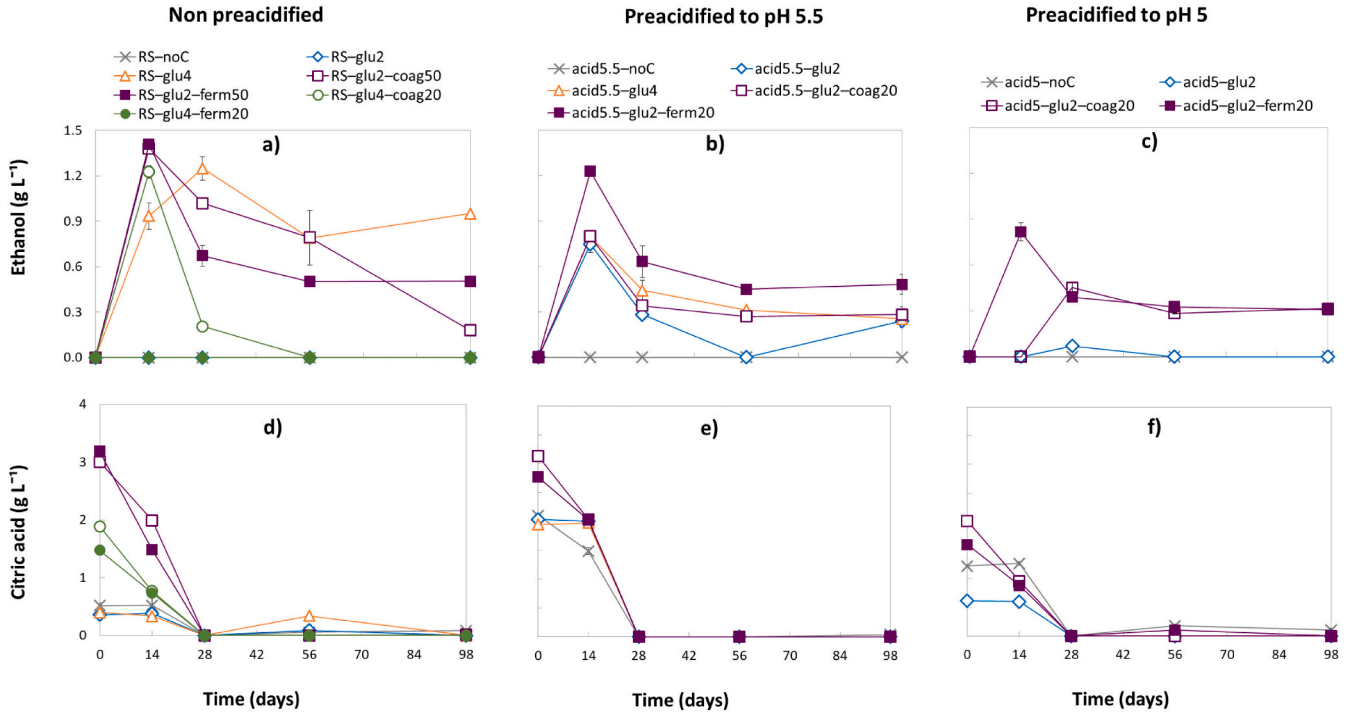

Fig. 5. Evolution in ethanol (above) and citric acid (below) concentration during the storage of treatments. Error bars represent the standard error of the mean (n = 3). For treatment abbreviations, see Table 1.

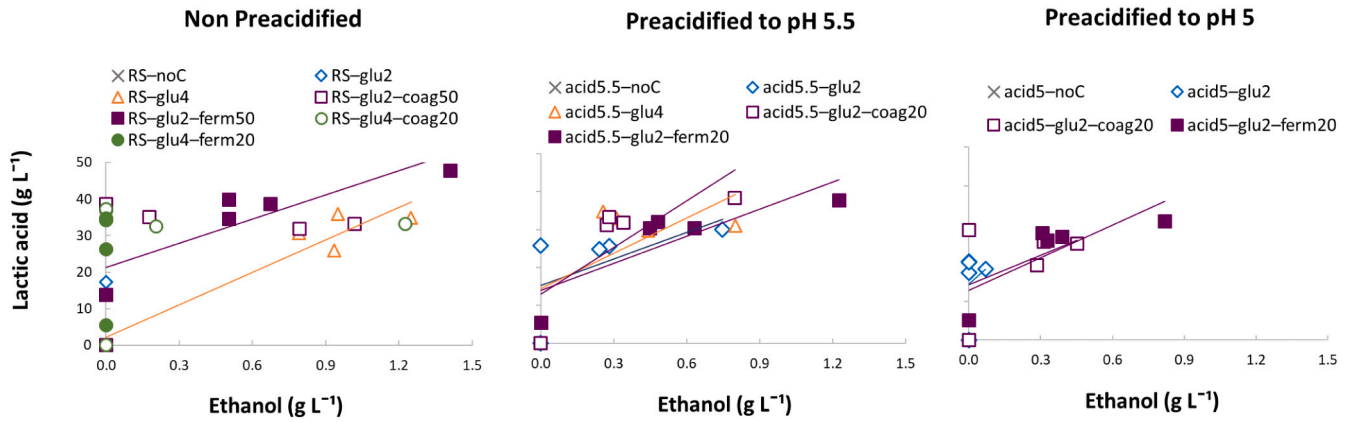

Fig. 6. Correlations between lactic acid and ethanol produced throughout the storage.

fermentation during the first two weeks of storage.

In summary, the maintenance of a low pH due to high lactic acid production and the avoidance of lactic acid conversion into other weaker VFAs, together with a reduction in CH_4 emission, suggest that RS-glu2-ferm50 was the optimal treatment. Similar results were observed with the acid5.5-glu4 treatment, but if the intention were to avoid acid usage, RS-glu2-ferm50 would be a more suitable option.

The ammonia (NH_3) that volatilises and pollutes the atmosphere could be retained in the bioacidified slurry as additional nitrogen, and would potentially result in an increase in agricultural mineral fertiliser equivalent value of the bioacidified slurry. However, very few studies have as yet been conducted on the fertiliser value of bioacidified slurry, so this still requires verification.

4. Conclusions

Bio-acidification of slurry by addition of fermentable substrates efficiently reduced and maintained low slurry pH via production of lactic acid. Lowering initial pH proved beneficial in initiating the fermentation process.

Efficient bioacidification occurred with minimum 2 % glucose addition together with a decreased initial pH, but higher glucose additions did not produce higher lactic acid concentrations.

By reaching a pH of 4.2, production of lactic acid was promoted and if substrate was still available, weaker VFAs were avoided, resulting in lower CH_4 emissions.

We conclude that bio-acidification could effectively replace sulfuric acid for reducing gaseous emissions during slurry storage.

CRediT authorship contribution statement

I. Regueiro: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Visualization, Writing – original draft. **B. Gómez-Muñoz:** Conceptualization, Investigation, Methodology, Supervision, Writing – review & editing. **M. Lübeck:** Resources. **M. Hjorth:** Conceptualization, Writing – review & editing. **L.**

Stoumann Jensen: Conceptualization, Funding acquisition, Methodology, Supervision, 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

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biteb.2022.101135.

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