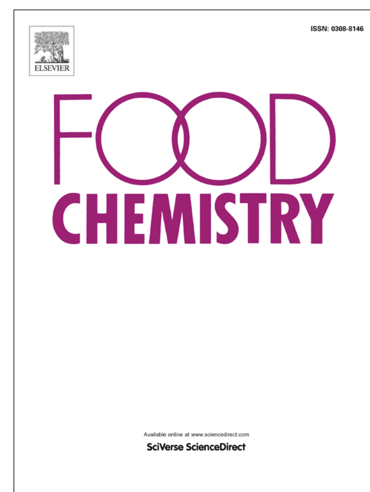
Accepted Manuscript



Acid production, growth kinetics and aroma profiles of *Lactobacillus* flora from Stilton cheese

Diriisa Mugampoza, Konstantinos Gkatzionis, Robert S.T. Linforth, Christine E.R. Dodd

PII: S0308-8146(19)30405-4
DOI: https://doi.org/10.1016/j.foodchem.2019.02.082
Reference: FOCH 24393

To appear in: *Food Chemistry*

Received Date: 1 October 2018
Revised Date: 20 February 2019
Accepted Date: 21 February 2019

Please cite this article as: Mugampoza, D., Gkatzionis, K., Linforth, R.S.T., Dodd, C.E.R., Acid production, growth kinetics and aroma profiles of *Lactobacillus* flora from Stilton cheese, *Food Chemistry* (2019), doi: https://doi.org/10.1016/j.foodchem.2019.02.082

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

ACCEPTED MANUSCRIPT

Acid production, growth kinetics and aroma profiles of *Lactobacillus* flora from Stilton cheese

Diriisa MUGAMPOZA[a,b*], Konstantinos GKATZIONIS[a,c], Robert S.T. LINFORTH[a], Christine E.R. DODD[a]

[a]Division of Food Sciences, University of Nottingham, Leicestershire LE12 5RD, UK, E-mails: robert.linforth@nottingham.ac.uk, christine.dodd@nottingham.ac.uk

[b]Department of Food Technology, Kyambogo University, P.O. Box 1, Kampala, Uganda, E-mail: mugampoza@gmail.com

[c]Department of Food Science and Nutrition, School of the Environment, University of the Aegean, Metropolite Ioakeim 2, GR 81400, Myrina, Lemnos, Greece, E-mail: kgkatzionis@aegean.gr

CORRESPONDENCE

Dr. Diriisa MUGAMPOZA

Tel: +256-781-713555

E-mail: mugampoza@gmail.com

Running Title: Aroma profile of *Lactobacillus* from Stilton Cheese

Manuscript length: 5,643 words

Number of figures: 03

ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

ABSTRACT

The effect of *Lactobacillus plantarum* isolates from Stilton cheese on aroma profiles of milk fermentation was examined. Representative *Lb. plantarum* isolates were cultured alone and in combination with acid-producing and non-acid producing *Lactococcus lactis* NCIMB 9918 in UHT milk at 30 & 18°C for 48 h & 12 weeks, respectively in presence and absence of salt, simulating cheese production and ripening. During long-term ripening, *Lb. plantarum* grew faster when co-cultured with non-acid producing *Lc. lactis* in the presence of salt. One isolate of *Lb. plantarum* produced the highest concentration of alcohols, organic acids and acetoin. Co-culture of *Lb. plantarum* with acid-producing *Lc. lactis* enhanced acid and alcohol production, whereas co-inoculation with non-acid producing *Lc. lactis* increased acetoin synthesis. *Lb. plantarum* is an incidental organism in cheese and its presence is unpredictable. Occurrence of different genotypes of *Lb. plantarum* could contribute to batch to batch variation in the cheese aroma characteristics.

Key words: Stilton; aroma; *Lactobacillus*; *Lactococcus*.

Highlights

- Fortuitous *Lactobacillus* affects aroma inconsistency during cheese production.
- *Lactobacillus* isolated from different sites in Stilton formed different volatiles.
- Co-culture of *Lactobacillus* with *Lactococcus lactis* enhanced aroma production.
- Salt had minimal effect on volatiles produced by *Lactobacillus* isolates.

ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

1. Introduction

Stilton is a protected designation origin (PDO) semi-soft blue-veined cheese produced in the counties of Nottinghamshire, Leicestershire and Derbyshire in the United Kingdom (Fernández, Mauriz, Gómez & Martín, 2009). The cheese is made from pasteurised cows' milk acidified by *Lactococcus lactis* subsp. *lactis* biovar. *diacetylactis* or *Lc. lactis* subsp. *cremoris* (Morgana, O'Sullivana, Rossa & Hill, 2002). *Penicillium roqueforti* is added to impart blueing and the typical sharp tangy flavor (Gkatzionis, Linforth & Dodd, 2009; Price et al., 2014; Ropars, Lopez-Villavicencio, Snirc, Lacoste & Giraud, 2017). The microbiota of Stilton has been investigated using conventional culture-dependent and molecular microbiological methods and mainly comprises lactobacilli, yeasts and moulds (Hiscox, Harrison & Wolf, 1940; Ercolini, Hill & Dodd, 2003).

Stilton, like other blue cheeses, has heterogeneous microenvironments with pronounced gradients of pH, salt, water activity and redox potential (Cantor, Van den Tempel, Hansen & Ardo, 2004; Fernández et al., 2009). Different sections of the cheese (outer crust, blue veins and white core) have considerable structural differences which influence the levels and distribution of gases within its matrix (Cantor et al., 2004). This strongly influences the growth, interaction and biochemical activity of inherent microbial communities at various stages of ripening (Martley & Crow, 1993). Ripening follows gradual microbial succession whereby acidification begins with *Lc. lactis* replaced by the more acid-tolerant lactobacilli (Broadbent, Houck, Johnson & Oberg, 2003; Martin-Platero, Valdivia & Maqueda, 2008), notably *Lactobacillus plantarum* (Hiscox et al., 1940; Whitley, 2002). *Lb. plantarum* is introduced as part of the microbial contaminants from raw milk, personnel and the processing plant environment (Hiscox et al., 1940). These organisms tolerate a range of pH, humidity, redox potential, salt and temperature (Martley & Crow, 1993; Broadbent et al., 2003), and interact with the starter *Lc. lactis* to produce lactate,

ACCEPTED MANUSCRIPT

diacetyl and acetate as the dominant aroma and antimicrobial compounds (Mugampoza, 2013). This effect is important in the prediction of product quality and safety and the knowledge is important in evaluation of non-starter *Lb. plantarum* with potential for development into commercial culture adjuncts for Stilton cheese.

Gkatzionis et al. (2009) studied the spatial distribution of aroma compounds in the outer crust, blue veins and white core of Stilton produced from different dairies and reported high heterogeneity of the compounds at different sites within a single cheese. Ketones were found to be concentrated in the outer crust and blue veins, whereas alcohols and aldehydes were mainly detected in the white core. Heterogeneity in the cheese aroma was confirmed in fermented milk under simulated cheese production conditions using *P. roqueforti* and *Lc. lactis* (starter cultures) in combination with *Yarrowia lipolytica*, the dominant secondary yeast microbiota in the cheese (Gkatzionis, Hewson, Hollowood, Hort, Dodd & Linforth, 2013).

Bacterial communities in the outer crust, blue veins and white core of Stilton are different in each section, comprising *Lc. lactis*, *Enterococcus faecalis*, *Lb. plantarum*, *Lb. curvatus*, *Leuconostoc mesenteroides*, *Staphylococcus equorum*, and *Staphylococcus* sp., with *Lb. plantarum* being the dominant species in all the cheese sections (Hiscox et al., 1940; Whitley, 2002; Ercolini et al., 2003). We have shown that in a single Stilton cheese there are a diverse range of *Lb. plantarum* strains present whose growth and survival characteristics are related to their site of isolation within the cheese (Mugampoza, 2013).There is therefore a need to establish the contribution of these fortuitously introduced *Lb. plantarum* in ripening and the typical aroma development of the cheese, including possible interactions between *Lb. plantarum* and the starter cultures that may affect the cheese aroma properties. In particular it is important to establish if these may contribute to the aroma differences observed between different sites within the cheese.

ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

This study examined the acidification capability of *Lb. plantarum* isolates obtained from the outer crust, blue veins and white core of the cheese, and assessed their influence on aroma production when co-cultured with different strains of *Lc. lactis* and with and without salt addition in a model medium. Isolates that were found to produce bacteriocins (Mugampoza, 2013) were assessed for antagonism to the primary starter culture *Lc. lactis*. The contribution of *Lb. plantarum* to the aroma profile of Stilton could be important for understanding the variation in the cheese quality characteristics from batch to batch.

2. Materials and methods

2.1. Chemicals

The culture media were obtained from Oxoid (United Kingdom). All chemicals used as standards for high performance liquid chromatography (HPLC) and aroma analysis were 99+% pure and purchased from Sigma-Aldrich (Gillingham, UK).

2.2. Source and preparation of the Stilton cheese sample

An 8 kg commercial sample of Stilton cheese at the end of ripening (45 days) was sourced from a local retailer in Nottingham and precisely partitioned into the outer crust, blue veins and white core under aseptic conditions. Micro-samples of each of the cheese section were prepared by aseptically scrapping 130-190 mg micro-samples into sterile o-ringed micro-centrifuge vials (Biospec Products, UK). The samples were mixed with nine parts of maximum recovery diluent (CM0733, Oxoid) and four glass beads (2 mm, acid washed, Biospec Products, UK), and homogenised using a Mini Beadbeater-1 (Biospec Products) at 2500 rpm for 2 x 40 s, cooling on ice between each treatment (Gkatzionis, 2010).

2.3. Isolation and identification of the isolates

ACCEPTED MANUSCRIPT

To isolate lactobacilli from the outer crust, blue veins and white core of a Stilton cheese, samples of the same cheese section were pooled and 10-fold serial dilutions prepared from 1 mL volumes of the pooled sample. The dilutions were plated on Rogosa agar (CM0627, Oxoid), and incubated anaerobically for 48 h at 30°C. After incubation, three to five colonies with different morphologies were randomly selected and streaked twice on Rogosa agar for purification (Gkatzionis, 2010).

All isolates (n=59) were Gram positive, catalase and oxidase negative rods and presumptively identified as *Lactobacillus* spp. (Mugampoza, 2013). Analysis of the 16S rDNA sequences obtained with the *Lactobacillus*-specific V6-V8 primers (Lopez et al., 2003) gave 97% of the isolates as *Lactobacillus plantarum* (3% were *Lb. brevis*). Sub-species typing and cluster analysis of the pulse-field gel electrophoresis (PFGE) patterns obtained with the enzyme *Not*I demonstrated a range of similarities between the isolates and five major geno-groups were delineated based on the site of isolation (Mugampoza, 2013). Subsequently, six representative *Lb. plantarum* isolates from the different cheese sites and five major PFGE clusters were selected to be examined for growth kinetics, aroma production and acidification capability.

2.4. Determination of lactic and acetic acid production

Lactobacillus plantarum isolates were grown in De Man Rogosa Sharpe (MRS) broth for 24 h at 30°C (Mugampoza, 2013). Then, 0.1 mL (6 $\log_{10}$ CFU/mL) was inoculated into 30 mL sterile MRS broth and incubated at 30°C, 100 rpm. Aliquots (5 mL) were withdrawn at different time intervals and centrifuged for 10 min at 3500 *g* to obtain cell-free supernatants (CFS). CFS were sterilised by membrane filtration (0.2 µm, Minisart AG37070, Sartorius, Germany). Organic acids were sampled using 30 mg Strata-X polymeric reverse solid phase extraction as described by Mugampoza (2013).

ACCEPTED MANUSCRIPT

Lactic and acetic acid were separated on an ion-exclusion Rezex ROA organic acid H^+ column phase (5 µm, 7.8 x 300 mm; Phenomenex, Macclesfield, UK) operated at ambient temperature with 0.005 N sulfuric acid as the mobile phase flowing at 0.5 mL/min, at a pressure of 50±5 kg/cm^2. The sample (10 µl) was injected into the HPLC using an auto-sampler (Jasco AS2055, Japan) and the acids detected using a refractive index detector (Jasco RI2031). Lactic and acetic acid were identified by matching the retention times with standards (Mugampoza, 2013). Quantification was achieved by linear integration of the data based on peak areas using Azur (1999-2005) software v.4.6 (Jasco). Mean values (g/L) were computed for individual organic acids after triplicate independent injections.

2.5. Growth kinetics of Lb. plantarum isolates

The kinetics of growth and survival of *Lactobacillus plantarum* was examined in ultra-high temperature treated (UHT) milk (3.5% fat) models. Individual isolates were grown alone or as co-cultures with an acid-producing wild strain of *Lactococcus lactis* (*Lc. lactis* subsp. *lactis,* University of Nottingham culture collection) or a non-acid producing *Lc. lactis* (*Lc. lactis* NCIMB 9918). The bacterial strains were spiked in 100 mL of the milk (final cell density, 3 $\log_{10}$ CFU/mL), and then incubated for 48 h at 30°C (Mugampoza, 2013). At 48 h, each sample was subdivided into two portions; salt (3.5%, w/v) was added to one, whilst the second portion (control) was un-salted. The samples were further incubated for 12 weeks at 18°C in order to simulate some of the cheese ripening conditions. The mixed lactic acid bacteria (LAB) cultures were differentially enumerated by incubating anaerobically for 48 h at 30°C on different selective media. Rogosa agar was used for enumeration of *Lb. plantarum* whereas *Lc. lactis* was differentially enumerated from MRS agar according to the method of Harris, Fleming and Klaenhammer (1992). MRS agar plates used for enumeration of lactococci in mixed culture (A) were replica

ACCEPTED MANUSCRIPT

plated on Rogosa agar to ascertain *Lb. plantarum* counts (B) and then worked out the difference (A-B) to obtain the result for *Lc. lactis*.

2.6. Aroma analysis

The aroma profiles of the samples were determined by solid phase micro-extraction gas chromatography-mass spectrometry (SPME GC-MS) according to the method of Gkatzionis (2010). Samples (5 mL) were obtained at different time intervals during growth (Section 2.4) and transferred into 20 mL headspace vials (22.5 mm x 75.5 mm, Grace Alltech, UK). The vials were immediately sealed with a PTFE-Silicone lined magnetic cap (20 mm diameter, 5 mm centre, Grace Alltech) and stored at -80°C until analysis. Samples were defrosted overnight at 4°C and allowed to equilibrate at room temperature (20°C) for 30 min prior to analysis. Three independent replicates were prepared for all samples.

A 1 cm Stableflex 50/30 μm SPME fibre with divinylbenzene-carboxen on polydimethylsilicone bonded to a flexible fused silica core (Supelco 5951, Bellefonte, USA) was used for extraction of the aroma volatiles. The fibre was first conditioned for 60 min in the injection port at 230°C. The SPME needle was introduced into the septum in the lid of the vial using a PAL auto-sampler (CTC Analytics, Switzerland) and the fibre exposed to the headspace for 20 min at room temperature. The temperature of the injection port was 230°C.

Chromatography was carried out with a Trace GC Ultra gas chromatograph (Thermo Electron Corporation, UK) using a 30 m Zebron ZB-5 capillary GC column (internal diameter 0.25 mm, film thickness 1 μm; Phenomenex, Macclesfield, UK). Helium gas was employed as the carrier gas at a constant pressure of 18 psi. The GC oven temperature programme was as follows: initial temperature was 40°C maintained for 2 min

ACCEPTED MANUSCRIPT

and increased at a rate of 8°C per min to a final temperature of 220°C. The transfer line from GC to MS was held at 250°C. MS was performed with a DSQ mass spectrometer (Thermo Electron Corporation) operating in positive ionisation electron impact mode (EI+) at 70 eV. The detector was operated in scan mode (2 scans/s) scanning from m/z 20-250. Source temperature was 200°C and pressure 39 mtorr. Identification was based on linear retention indices and mass spectra matches with those published in the National Institute of Standards and Technology (NIST) mass spectral library (Gkatzionis et al., 2009). The signal intensity (peak area) for each compound was expressed relative to that observed when the headspace above a 5 µg/L 2-nonanone solution prepared using de-ionised water (Gkatzionis et al., 2009) was sampled and data processed with Xcalibur software v.14 (Thermo Fisher Scientific, UK).

2.7. Statistical analysis

Microbial counts were normalised by conversion to $\log_{10}$ CFU/mL. Means, standard errors and Pearson's correlation coefficient (R) were computed using the Predictive Analytical Software (PASW), v.19. The effect of salt and different strains of *Lactococcus lactis* on aroma profiles of *Lactobacillus plantarum* isolates was evaluated by principal component analysis (PCA) using Unscrambler v.9.0 (Camo Process AS., Norway).

3. Results and discussion

3.1. Lactic and acetic acid production

Table 1 shows the growth, pH changes and acid production of *Lactobacillus plantarum* isolates over 48 h. Exponential growth phase was observed at 9-24 h, during which a noticeable pH decrease was detected due to concomitant lactic acid production. Further pH decrease (24-48 h) was recorded to the lowest value of 3.76±0.01 from *Lb.*

ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

plantarum W30 (isolated from the white core of the cheese), with consequent inhibition of cell growth. Lactic acid production was associated with the growth phase profile (*R*=0.75-0.85, *p*<0.05), and inversely with the pH changes (*R*=0.93-0.98, *p*<0.05). At 0-9 h, there was minimal lactic acid production for all the tested isolates, due to slow growth. Between 24-48 h however, maximal lactic acid production was recorded for all the isolates which is why this phase was mainly associated with the greatest pH decrease. At 48 h, *Lb. plantarum* W30 produced the highest level of lactic acid (21.40±1.5 g/L), whereas *Lb. plantarum* R2 (isolated from the outer crust) had the lowest (13.63±0.8 g/L). All isolates produced low levels (0.15 to 1.75 g/L) of acetic acid throughout the experiment. However, the concentration of acetic acid also showed a positive correlation with the growth phase (*R*=0.58-0.81, *p*<0.05), and an inverse relationship with pH (*R*=0.77-0.93, *p*<0.05). At 48 h, the lowest proportion of acetic acid (0.71±0.2 g/L) was obtained from *Lb. plantarum* R40 (white core), whereas the highest (1.75±0.5 g/L) was produced by R2 (outer crust).

This study has shown that lactic acid production in an aerated MRS medium correlates with cell numbers and is greatest at 48 h. The process was not inhibited by low pH, which is in agreement with reports by other authors (Passos, Fleming, Ollis, Felder & Mcfeeters, 1994; Schepers, Thibault & Lacroix, 2002). The greatest lactic acid production led to a pH drop of around 3 units and was largely dependent on the site in the cheese from which the isolates were obtained as demonstrated by the differences in pH levels recorded. The isolates from the white core produced significantly more lactic acid than those from the outer crust (*p*<0.05). However, the relationship between cell growth and acetic acid production was weaker, highlighting the known homofermentative nature of *Lb. plantarum* (Whitley, 2002; Mugampoza, 2013). While several studies have confirmed the validity of the linear relationship between lactic acid production and growth rate in most lactobacilli (Fu & Mathews, 1999; Narendranath & Power, 2005), interpretation of this

ACCEPTED MANUSCRIPT

relationship has been sometimes questionable (Passos et al., 1994). Therefore, whereas the current study has indicated a positive correlation between the density of *Lb. plantarum* cells and lactic acid production in MRS broth, in the real cheese matrix, growth and accompanying lactic acid production may show some variation depending on a number of factors: physiological state of cells, differences in carbon and nitrogen sources as well as the presence or absence of other growth factors (Cogan, Beresford, Steele, Broadbent, Shah & Ustunol, 2007). Differences in microenvironment and other microbiota within the cheese matrix (white core, blue veins or outer crust) could also influence growth and acid production (Giraffa & Carminati, 2008). In *Lb. delbrueckii* for example, addition of glucose and supplementation with yeast extracts enhances lactic acid production (Arasaratnam, Senthuran & Balasubraman, 1996). In *Enterococcus faecalis*, fermentation efficiency and lactic acid productivity is enhanced by protein hydrolysis. The proteolytic and lipolytic activity of *P. roqueforti* and yeasts including *Yarrowia lipolytica* and *Debaromyces hansenii* in Stilton cheese (Gkatzionis et al., 2009) could enhance lactic acid production in *Lb. plantarum* isolates, if grown in the cheese matrix. This may contribute to its flavour (Ammor, Tauveron, Dufour & Chevallier, 2006) as well as inhibit the growth of undesirable organisms (Gill & Newton, 1982). During early ripening (0-6 weeks) for the majority of blue cheeses, most of the lactic acid occurs in L (+) form (Passos et al., 1994). After piercing at 6-7 weeks, yeasts and moulds produce ammonia by deamination and decarboxylation of free amino acids, which subsequently increases the pH (Prieto, Urdiales, Franco, Tornadijo, Fresno & Carballo, 1999). This process contributes to development of cheese rheological properties (Gkatzionis, 2010).

3.2. Growth kinetics

The current work aimed to establish how the possible population interactions (competitive, synergistic or mutuality) between the *Lactobacillus plantarum* isolates and

different strains of *Lactococcus lactis* could influence the formation of aroma compounds in milk. As there was little difference in acidification capabilities of *Lactobacillus* isolates from a given cheese site (Table 1), one representative isolate from each site was chosen to examine the differences in aroma properties of *Lactobacillus* isolates obtained from the different sites. Isolate R2 was selected from the outer crust, B30 from the blue veins and W8 from the white core. Two different *Lc. lactis* strains were used in this work: acid-producing *Lc. lactis* subsp. *lactis* (LcA) and non-acid producing *Lc. lactis* NCIMB 9918 (LcNA).

3.2.1. Early fermentation at 30°C

During early fermentation (0-48 h), *Lactobacillus plantarum* isolates grown alone had similar growth characteristics (Fig. 1). In all instances, LcA alone or in mixed culture attained higher final counts than LcNA and *Lb. plantarum* isolates. In mixed culture, there were differences in final cell numbers for *Lb. plantarum* isolates; *Lb. plantarum* was reduced in the presence of LcA compared with singlely grown strains, and further reduced by LcNA compared with LcA. *Lb. plantarum* B30 grew to a higher cell density when co-cultured with LcA than other *Lactobacillus* isolates. The isolate also stimulated more growth of *Lactococcus lactis* strains in mixed culture when compared with the isolates from the outer crust and white core (Mugampoza, 2013; Fig. 7.5). Therefore, this difference could be attributed to a better competitiveness of this isolate rather than it having an inherently greater growth rate than *Lb. plantarum* R2 and W8 (Mugampoza, 2013). Both *Lc. lactis* strains grew rapidly to reach 8-9 $\log_{10}$ CFU/mL at 12-48 h. The viable counts for LcA remained stable at this level, whereas those of LcNA alone or in mixed culture gradually decreased to 6-7 $\log_{10}$ CFU/mL. Overall, the results suggested that during early fermentation, growth of *Lb. plantarum* isolates in milk was suppressed by *Lc. lactis*. *Lb. plantarum* B30 grew better in the presence of the acid-producing *Lc. lactis* than R2 and

ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

W8, adapting to attain a final viable count similar to that obtained in single culture. However, this effect was not demonstrable when the non-acid producing *Lc. lactis* was used, implying other factors could account for the reduced growth of the *Lactobacillus* isolate when co-cultured with LcNA. It was also noticeable that pH changes during early fermentation were mainly caused by growth of acid-producing *Lc. lactis* rather than non-acid producing *Lc. lactis* or *Lb. plantarum* isolates (data not shown). The results were congruent with HPLC data (Section 3.1) and highlighted the slow acidification capabilities of the *Lb. plantarum* isolates.

3.2.2. Long-term ripening at 18°C

During long-term ripening, the viable counts of non-acid producing *Lactococcus lactis* (LcNA) and *Lactobacillus plantarum* isolates alone or in mixed cultures remained stable at 6-7 and 7-8 log_{10} CFU/mL, respectively (Fig. 2A, 2C and 2E). The viable counts of acid producing *Lc. lactis* (LcA) grown alone were undetectable at 12 weeks of ripening (Fig. 2A), whereas this *Lactococcus* strain was undetectable in mixed culture after week 7 (Fig 2D; detection limit, 3 log_{10} CFU/mL). Co-culture of *Lb. plantarum* B30 with LcA was more inhibitory for the growth and survival of the *Lc. lactis* than other treatments, which may be due to higher acidity of the medium or due to stimulation of this *Lactobacillus* isolate to produce antimicrobial compounds in response to the pH drop (Mugampoza, 2013). Although results of early fermentation indicated that LcNA was more antagonistic for *Lb. plantarum* isolates than LcA, incubation at 18°C subsequently enhanced the growth of lactobacilli in co-culture with LcNA (Fig 2C).

At 12 weeks of ripening, the addition of salt had no significant effect on the growth characteristics of *Lactobacillus* isolates alone or in mixed culture (Fig. 2A-E; p>0.05). In comparison, salt had variable effects on the *Lc. lactis* strains; its presence did

not cause significant reduction in viable counts of LcNA grown alone or in mixed culture (Fig. 2A; $p>0.05$). Salt showed significant inhibition of the growth of LcA ($p<0.05$); this effect was much greater in co-culture (Fig. 2D) showing that the main factor which influenced *Lc. lactis* survival was acid production, as LcA showed much poorer survival when pH was lowest. Hence pH, and not salt, appears to be the main factor causing the *Lc. lactis* starter to die off in maturing cheeses.

By the end of ripening at 12 weeks, *Lb. plantarum* isolates dominated the microbiota of mixed cultures which confirmed our hypothesis that *Lc. lactis* which is included in the cheese as a primary starter culture disappears from the fermentation profile by the end of ripening. The contribution of lactic acid bacteria in blue cheese ripening occurs prior to piercing at 6-7 weeks, after which *Penicillium roqueforti* dominates the cheese matrix up to the end of the process (Ercolini et al., 2003). This study has shown that up to seven weeks, both *Lc. lactis* and *Lb. plantarum* remain viable and therefore potentially participate in substrate metabolism within the cheese matrix.

At 48 h at 30°C, the viable count of *Lb. plantarum* isolates alone and in mixed culture (5.8-7.5 $\log_{10}$ CFU/mL) were lower than those of *Lc. lactis* (7.4-9.0 $\log_{10}$ CFU/mL) (Fig. 1). The latter corresponds to the number (>10^9 CFU/mL) usually found in freshly prepared cheese curds (Broadbent et al., 2003). During 12 weeks of ripening at 18°C, the population of *Lb. plantarum* isolates in co-culture with LcA remained stable at 7 $\log_{10}$ CFU/mL (Fig. 2B) while that of the *Lc. lactis* rapidly declined to undetectable levels (Fig. 2D).These results are in agreement with the studies of Martin-Platero et al. (2008) who pointed out that *Lactobacillus* persists at different stages of ripening to become the dominant LAB component of ripened cheese. Changes in relative humidity (Hay, 2017), redox potential, salt concentration, pH, presence or absence of nutrients are regarded as major factors contributing to the microbial succession dynamics favouring the dominance

ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

of lactobacilli in ripened cheese (Broadbent et al., 2003; Martin-Platero et al., 2008; Martley & Crow, 1993).

Death of LcA (Fig. 1-2) could be attributed to autolysis due to low pH (Gatti et al., 2008). The process is enhanced by salt, low temperature, water activity and high acidity (Ramírez-Nuñez, Romero-Medrano, Nevárez-Moorillón & Gutiérrez-Méndez, 2011), which may explain our results. However, the current study was performed in a simple UHT milk model. Therefore, the actual growth, survival, and activity of *Lb. plantarum* isolates *in situ* in Stilton cheese may be determined by co-presence of other microbial species and *in situ* cell-to-cell interactions which often happen in a solid phase system (Giraffa, 2004; Giraffa & Carminati, 2008). This aspect needs further investigation for each of the *Lactobacillus* isolates examined in this study. Research on the impact of non-starter lactobacilli on cheese flavour is vital for identification of strains with potential as adjunct cultures (Wouters, Ayad, Hugenholtz & Smit, 2002). We have, therefore, partly investigated this aspect for some of the *Lactobacillus* isolates (Section 3.3).

3.3. Aroma profiles by SPME GC-MS

3.3.1. Early fermentation

The effect of growth interactions obtained from Section 3.2, on the aroma profile of milk was examined using headspace analysis. The SPME GC-MS chromatograms consisted of 21 main peaks, including six ketones, five alcohols, four esters, three carboxylic acids and three aldehydes. At 48 h, the aroma profile of *Lactobacillus plantarum* isolates alone and that of their co-culture with non-acid producing *Lactococcus lactis* (LcNA) were similar to that of the un-inoculated milk control (Table 2). Alcohols and aldehydes were detected in the sample inoculated with acid producing *Lc. lactis* (LcA) alone. The levels were amplified when non-acid producing *Lc. lactis* (LcNA) was co-

ACCEPTED MANUSCRIPT

cultured with *Lb. plantarum* B30, whereas co-culture of LcA with *Lb. plantarum* R2 and W8 gave the same level of these compounds as LcA alone.

At 48 h, 3-methyl butanol (62±4 μg/l) and 2-methyl butanol (16.4±0.5 μg/l) were the dominant alcohols detected in the B30/LcA co-culture. Two-methyl butanal (14±1 μg/l) and 3-methyl butanal (47±3 μg/l) were the dominant aldehydes associated with the B30/LcA culture. Acetoin was mainly found (2.6±0.9 μg/l) with LcNA either alone or in co-culture with *Lb. plantarum* isolates.

3.3.2. Long-term ripening

At seven weeks, single and mixed cultures of *Lactobacillus plantarum* isolates produced various groups of volatiles (Fig. 3). The high level of aldehydes detected with the LcA – *Lb. plantarum* B30 co-culture at 48 h (Table 2) could have been reduced into corresponding alcohols at seven weeks (Vítová, Loupancová, Zemanová, Štoudková, Březina and Babák, 2006). Principal component analysis scores and loadings bi-plot of the data showed significant variability among the samples ($p<0.05$; Fig. 3). LcA in single culture produced significant levels of 2- and 3-methyl butanal prior to cell death as shown in Section 3.2. The aroma profiles of *Lb. plantarum* R2 and W8, and LcNA alone or in co-culture were generally similar to that of uninoculated milk and comprised hexanol, propanoic acid methyl ester and 3-methyl butanal. Acetic acid was mostly produced by *Lb. plantarum* B30. Co-culture of *Lactobacillus* isolates with LcA, and single or mixed cultures of *Lb. plantarum* B30 with LcNA amplified synthesis of acetoin, alcohols and organic acids. Symbiosis was mainly evident in the production of acetic acid, 3-methyl butanol, 2-pentanone, 2-hexanone and butanol 3-methyl acetate. In this context, acid production was mostly enhanced in co-cultures of either of the *Lc. lactis* strains with *Lb. plantarum* R2 and W8, whereas alcohol synthesis was enhanced in their co-culture with *Lb. plantarum* B30. Ketone synthesis was enhanced in all co-cultures, while aldehyde production was mainly

ACCEPTED MANUSCRIPT

enhanced in the co-culture of *Lb. plantarum* B30 with LcA. Salt addition had minimal effect on the distribution of volatiles.

Data from the current work have shown that, in contrast to the usual description of *Lb. plantarum* as homofermentative (Whitley, 2002; Mugampoza, 2013), the isolates which were examined were facultatively heterofermentative mainly producing lactic acid as observed in Section 3.1 but with some production of acetic acid, 2-methyl propanol and acetoin. These compounds were detected at higher levels with the *Lb. plantarum* single cultures than un-inoculated milk control at 7 weeks (data not shown). Mukisa, Byaruhanga, Muyanja, Langsrud and Narvhus (2016) reported that *Lb. plantarum* produces acetic acid, acetaldehyde, diacetyl, and acetoin which is congruent with our results. Årskold, Lohmeier-Vogel, Cao, Roos, Rådstrom and Van Niel (2008) suggested that the heterofermentative process in *Lactobacillus* normally occurs in the presence of low oxygen, which corresponds with the conditions (sealed vials) under which the current study was conducted.

Previous studies on blue cheese varieties manufactured from pasteurised milk have found high levels of carbonyl compounds, methyl ketones (Price et al., 2014), alcohols, carboxylic acid and aldehydes at the end of ripening (Lawlor, Delahunty, Sheehan & Wilkinson, 2003; Vítová et al., 2006). Gkatzionis et al. (2009) showed that different zones in Stilton cheese contain different levels of aroma volatiles. In general, the outer crust and blue veins contained ketones, the outer crust was associated with methanethiol, dimethyl-disulfide, ethanol, a-pinene and 3-methyl-2-pentanone whereas the white core contained alcohols and aldehydes as the the major compounds. These authors indicated that this association varies, to some extent, with the dairy of origin of the cheese.

The current study has shown that *Lb. plantarum* isolates can contribute substantial amounts of acid, alcohol, aldehyde and ketone (specifically acetoin)

ACCEPTED MANUSCRIPT

compounds during cheese ripening and the levels were partly dependent on the sites from which the isolates were obtained showing they were genetically different strains of *Lb. plantarum* occurring within a single Stilton cheese. This has been substantiated by subspecies typing studies (manuscript in preparation). Alcohols, organic acids and carbonyls generally increased with time. However, levels of most of the individual ketones at progressive stages of ripening showed some fluctuation and in some cases inversely correlated with their corresponding alcohols and aldehydes due to biochemical inter-conversion (Madkor, Fox, Shalabi & Metwalli, 1987).

The dominant volatiles at 7 weeks were ketones (principally acetoin), organic acids (acetic, hexanoic and butanoic acid) and alcohols including 3-methyl butanol and 2-methyl propanol. In single culture, these compounds were mainly produced by *Lb. plantarum* B30 which was isolated from the blue veins and therefore from one of the sites shown to have high ketone levels by Gkzationis et al. (2009). However, the correspondence of site of origin of each *Lactobacillus* isolate and its volatiles production did not show a strong association with the main volatiles reported at each site by Gkatzionis et al. (2009) showing the likely impact of other members of the Stilton microbiota (Gkatzionis et al., 2013).

Lawlor, Delahunty, Sheehan and Wilkinson (2003) reported that methyl ketones are responsible for the unique flavour of blue cheeses. The current study showed that *Lb. plantarum* can contribute to these aroma notes in Stilton cheese. *Lb. plantarum* B30 also produced higher levels of 2- and 3-methyl butanal and acetoin (buttery note) than others, as well as 2-methyl butanol and 3-methyl butanol, which have fruity, alcoholic and malty notes (Gkatzionis et al., 2009). These branched alcohols result from biochemical reduction of corresponding methyl ketones (Lawlor et al., 2003). Co-culture of *Lb. plantarum* B30 with *Lc. lactis* and salt addition stimulated acetoin and alcohol synthesis. Although strains

ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

of *Lc. lactis* are included in Stilton cheese as a starter culture, from Section 3.2, the acid-producing *Lc. lactis* (LcA) strain could not be detected at the seventh week whereas the non-acid producing counterpart (LcNA) and *Lb. plantarum* were still viable. From our data, the volatiles produced by LcA could persist in the medium whereas others could be amplified by continued growth of *Lb. plantarum* in co-culture. This was the case for 2-methyl propanal and 2- & 3-methyl butanal for LcA single cultures, as well as 2-methyl propanol, 2-methyl butanol and 3-methyl butanol in the LcA mixed cultures with *Lb. plantarum* B30.

Our study has demonstrated that *Lb. plantarum* could, as expected, grow and remain viable in fermented milk for 12 weeks at low temperature and low pH. Salt addition had minimal effect on the growth and volatile production characteristics of the organism irrespective of the isolate of *Lb. plantarum* or the strain of *Lc. lactis* with which it was co-cultured. At 48 h, *Lb. plantarum* produced lactic acid and low levels of acetic acid and this depended on growth phase as well as the particular isolate of *Lactobacillus* examined. Aroma studies in milk also revealed marginal levels of acetic acid produced between 24-48 h but synthesis was enhanced during longer incubation at 18°C. From this account, it could be concluded that, during early fermentation, *Lb. plantarum* predominantly produces lactic acid. However, during long-term ripening, the organism metabolises the acid and other substrates to produce acetic acid, ethanol and acetoin (Singh, Drake & Cadwallader, 2003) as evidenced in 3.3.2. As these volatiles have a positive contribution to the aroma profile of fermented milk (Vítová et al., 2006), *Lb. plantarum* isolates examined in our study may be considered to contribute positively to the fermentation and thus could be suitable for inclusion as starter culture adjuncts during the production of Stilton and other blue cheeses. As Gkatzionis et al. (2009) reported ketones and alcohols to be the major compounds in Stilton cheese, it is possible that *Lb. plantarum* makes a substantial

ACCEPTED MANUSCRIPT

contribution to these compounds in the cheese. However, the volatiles produced by specific *Lactobacillus* isolates did not always correlate with the known volatiles shown to predominate in the section of cheese from which they were isolated.

Stilton has a complex fungal flora mainly comprising *Penicillium roqueforti* and yeasts (Whitley, 2002; Gkatzionis et al., 2009). Most of these organisms directly produce aroma compounds but also indirectly contribute to the cheese flavour through proteolysis and lipolysis (Lawlor et al., 2003; Gkatzionis et al., 2009). Further research should investigate the profile of compounds produced in Stilton as a result of symbiotic interactions between different *Lb. plantarum* isolates and fungal communities. There is the need to determine the critical levels (or ratios) of the important microbial species whose interaction is of significance to the flavour properties of Stilton. Overall, it was clear from our study that co-presence of some *Lb. plantarum* isolates and *Lc. lactis* strains results in increased synthesis of alcohols, organic acids and ketones normally present in high quality Stilton (Gkatzionis et al., 2009), and as such could be a major contributory factor to the differences in aroma profiles of cheeses manufactured within similar or different batches and/or creameries. This understanding could be utilised to improve the aroma profile of Stilton especially if the synergistic effect of *Lb. plantarum* and *Lc. lactis* in synthesis of these compounds can be confirmed *in situ* in the real cheese matrix.

Individual strains can pose profound effects on cheese flavour and body characteristics (Broadbent et al., 2003) and it was shown in the current study that volatile production was dependent on the specific *Lb. plantarum* isolate. The unpredictable and dynamic nature of the presence of different strains of *Lb. plantarum* in Stilton could therefore be an important source of cheese flavour defects and production inconsistencies. This hypothesis has been supported by results of this study. In fact, stringent control of non-starter organisms during cheese ripening in order to produce more consistent high

ACCEPTED MANUSCRIPT

quality products has been less successful (Broadbent et al., 2003). The *Lb. plantarum* isolates evaluated in this study could, therefore, be used as an adjunct culture for Stilton as a strategy to accomplish this goal.

It has been suggested that a good culture adjunct should have good growth characteristics, antimicrobial activity, acid production, compatibility with the cheese starter cultures, ability to tolerate the stress conditions in cheese and make a positive contribution to the volatile aroma profile of the cheese (Crow, Curry & Hayes, 2001) which have been demonstrated to different degrees by our isolates. These efforts are supported by Kocaoglu-Vurma, Harper, Drake & Courtney (2008) who suggested that flavour development of ripened cheese can be enhanced using non-starter lactic acid bacteria dominated by wild strains of lactobacilli. As *Lb. plantarum* has been successfully applied as a culture adjunct in Swiss (Kocaoglu-Vurma et al., 2008) and Cheddar cheeses (Crow et al., 2001), its prospects for this application in Stilton are worth further investigation.

4. Conclusion

This study showed that during early fermentation, lactic acid was the major metabolite produced by all *Lactobacillus plantarum* isolates and its final concentration was a function of cell density achieved during the growth phase and was insensitive to lactic acid end product inhibition. During long term ripening, co-culture of *Lb. plantarum* with different strains of *Lactococcus lactis* enhanced aroma production. As lactobacilli are fortuitously introduced organisms, it was concluded that aroma production is largely dependent on type of strains entering into the cheese during production. Overall, this would lead to variation in product characteristics from batch to batch.

ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

Conflict of Interest Statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Acknowledgements

This work was supported by Islamic Development Bank (Grant No: 27528-94062645) and Kyambogo University, Uganda. Authors are grateful to Mr. Stewart Wilkinson, Ola and Dr. Andrew Timms for their technical assistance.

References

Ammor, S., Tauveron, G., Dufour, E., & Chevallier, I. (2006). Antibacterial activity of lactic acid bacteria against spoilage and pathogenic bacteria isolated from the same meat small-scale facility 1: Screening and characterisation of the antibacterial compounds. *Food Control, 17*, 454-461.

Arasaratnam, V., Senthuran, A., & Balasubramaniam, K. (1996). Supplementation of whey with glucose and different nitrogen sources for lactic acid production by *Lactobacillus delbrueckii*. *Enzyme and Microbial Technology, 19*, 482-486.

Årskold, E., Lohmeier-Vogel, E., Cao, R., Roos, S., Rådstrom, P., & van Niel, E. W. J. (2008). Phosphoketolase pathway dominates in *Lactobacillus reuteri* ATCC 55730 containing dual pathways for glycolysis. *Journal of Bacteriology, 190*, 206-212.

ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

Broadbent, J. R., Houck, K., Johnson, M. E., & Oberg, C. J. (2003). Influence of adjunct use and cheese microenvironment on nonstarter bacteria in reduced-fat Cheddar-type cheese. *Journal of Dairy Science, 86*, 2773-2782.

Cantor, M. D., van den Tempel, T., Hansen, T. K., & Ardo, Y. (2004). Blue cheese. In: Fox, P. F., McSweeney, P. L. H., Cogan, T. M., & Guinee, T. P. (Eds). Cheese: chemistry, physics and microbiology. 3rd edition, volume 2 (Major cheese groups). *Elsevier Academic Press, London, UK*, 1-438.

Cogan, T. M., Beresford, T. P., Steele, J., Broadbent, J., Shah, N. P., & Ustunol, Z. (2007). *Invited Review:* Advances in starter cultures and cultured foods. *Journal of Dairy Science, 90*, 4005-4021.

Crow, V., Curry, B., & Hayes, M. (2001). The ecology of non-starter lactic acid bacteria (NSLAB) and their use as adjuncts in New Zealand Cheddar. *International Dairy Journal, 11*, 275-283.

Ercolini, D., Hill, P. J., & Dodd, C. E. R. (2003). Bacterial community structure and location in Stilton cheese. *Applied and Environmental Microbiology, 69*(6), 3540-3548.

Fernández, M. A. B., Mauriz, E., Gómez, A., & Martín, J. F. (2009). Proteolytic activity, mycotoxins and andrastin A in *Penicillium roqueforti* strains isolated from Cabrales, Valdeón and Bejes–Tresviso local varieties of blue-veined cheeses. *International Journal of Food Microbiology, 136*, 18-25.

Fu, W., & Mathews, A. P. (1999). Lactic acid production from lactose by *Lactobacillus plantarum*: kinetic model and effects of pH, substrate, and oxygen. *Biochemical Engineering Journal, 3*, 163-170.

Gatti, M., Lindner, J. D., De Lorentiis, A., Bottari, B., Santarelli, M., Bernini, V., & Neviani, E. (2008). Dynamics of whole and lysed bacterial cells during Parmigiano-

ACCEPTED MANUSCRIPT

Reggiano cheese production and ripening. *Applied and Environmental Microbiology, 74*, 6161-6167.

Gill, C. O., & Newton, K. G. (1982). Effect of lactic acid concentration on growth on meat of Gram negative psychrotrophs from a meat works. *Applied and Environmental Microbiology, 43*, 284-288.

Giraffa, G. (2004). Studying the dynamics of microbial populations during food fermentation. *FEMS Microbiology Reviews, 28*, 251-260.

Giraffa, G., & Carminati, D. (2008). In: Cocolin, L., & Ercolini, D. (Eds). Molecular techniques in food fermentation: Principles and applications. *Springer Science and Business Media, New York, USA*, 1-4.

Gkatzionis, K., Linforth, R. S. T., & Dodd, C. E. R. (2009). Volatile profile of Stilton cheeses: Differences between zones within a cheese and dairies. *Food Chemistry, 113*, 506-512.

Gkatzionis, K. (2010). *Flavour production of Stilton blue cheese microflora. Thesis*. The University of Nottingham.

Gkatzionis, K., Hewson, L., Hollowood, T., Hort, J., Dodd, C. E. R., & Linforth, R. S. T. (2013). Effect of *Yarrowia lipolytica* on blue cheese odour development: Flash profile sensory evaluation of microbiological models and cheeses. *International Dairy Journal, 30*, 8-13.

Hay, C. (2017). The effect of humidity controlled environment on Stilton cheese. *International Dairy Journal*, 70, 1-5.

Kocaoglu-Vurma, N. A., Harper, W. J., Drake, M. A., & Courtney, P. D. (2008). Microbiological, chemical, and sensory characteristics of Swiss cheese manufactured with adjunct *Lactobacillus* strains using a low cooking temperature. *Journal of Dairy Science, 91*, 2947-2959.

ACCEPTED MANUSCRIPT

Harris, L. J., Fleming, H. P., & Klaenhammer, T. R. (1992). Novel paired starter culture system for Sauerkraut, consisting of a nisin-resistant *Leuconostoc mesenteroides* strain and a nisin-producing *Lactococcus lactis* strain. *Applied and Environmental Microbiology, 58*, 1484-1489.

Hiscox, E. R., Harrison, J., & Wolf, J. Z. (1940). The microflora of Stilton cheese. *Journal of Applied Microbiology (2008) 3*, 23-26.

Lawlor, J. B., Delahunty, C. M., Sheehan, J., & Wilkinson, M. G. (2003). Relationships between sensory attributes and the volatile compounds, non-volatile and gross compositional constituents of six blue-type cheeses. *International Dairy Journal, 13*, 481-494.

Lopez, I., Ruiz-Larrea, F., Cocolin, L., Orr, E., Phister, T., Marshall, M., Gheynst, J.V. and Mills, D.A. (2003) Design and evaluation of PCR primers for analysis of bacterial populations in wine by denaturing gradient gel electrophoresis. *Applied and Environmental Microbiology* 69, 6801-6807.

Madkor, S., Fox, P. F., Shalabi, S. I., & Metwalli, N. H. (1987). Studies on the ripening of Stilton cheese: Lipolysis. *Food Chemistry, 25*, 93-109.

Martin-Platero, A. M., Valdivia, E., & Maqueda, M. (2008). Polyphasic approach to bacterial dynamics during the ripening of Spanish farmhouse cheese, using culture dependent and independent methods. *Applied Environmental Microbiology, 74*, 5662-5673.

Martley, F. G., & Crow, V. L. (1993). Interactions between non-starter microorganisms during cheese manufacture and ripening. *International Dairy Journal, 3*(4-6), 461-483.

ACCEPTED MANUSCRIPT

Morgana, S. M., O'Sullivana, L., Rossa, R. P., & Hill, C. (2002). The design of a three strain starter system for Cheddar cheese manufacture exploiting bacteriocin-induced starter lysis. *International Dairy Journal 12,* 985-993.

Mugampoza, D. (2013). *Characterisation of the non-starter bacterial flora of Stilton cheese. Thesis*. The University of Nottingham.

Mukisa, I. M., Byaruhanga, Y. B., Muyanja, C.M.B.K., Langsrud, T., & Narvhus, J. A. (2016). Production of organic flavor compounds by dominant lactic acid bacteria and yeasts from *Obushera*, a traditional sorghum malt fermented beverage. *Food Science and Nutrition, 5,* 702-712.

Narendranath, N. V., & Power, R. (2005). Relationship between pH and medium dissolved solids in terms of growth and metabolism of lactobacilli and *Saccharomyces cerevisiae* during ethanol production. *Applied and Environmental Microbiology, 71*, 2239-2243.

Passos, F. V., Fleming, H. P., Ollis, D. F., Felder, R. M., & Mcfeeters, R. F. (1994). Kinetics and modelling of lactic acid production by *Lactobacillus plantarum*. *Applied and Environmental Microbiology, 60*, 2627-2636.

Price, J. E., Linforth, R. S. T., Dodd, C. E. R., Phillips, C. A., Hewson, L., Hort, J., & Gkatzionis, K. (2014). Study of the influence of yeast inoculum concentration (*Yarrowia lipolytica* and *Kluyveromyces lactis*) on blue cheese aroma development using microbiological models. *Food Chemistry*, 145, 464-472.

Prieto, B., Urdiales, R., Franco, I., Tornadijo, M. E., Fresno, J. M., & Carballo, J. (1999). Biochemical changes in PicoÂ n Bejes-Tresviso cheese, a Spanish blue-veined variety, during ripening. *Food Chemistry, 67*, 415-421.

ACCEPTED MANUSCRIPT

Ramírez-Nuñez, J., Romero-Medrano, R., Nevárez-Moorillón, G. V., & Gutiérrez-Méndez, N. (2011). Effect of pH and salt gradient on the autolysis of *Lactococcus lactis* strains. *Brazilian Journal of Microbiology, 42*, 1495-1499.

Ropars, J., Lopez-Villavicencio, M., Snirc, A., Lacoste, S., & Giraud, T. (2017). Blue cheese-making has shaped the population genetic structure of the mould *Penicillium roqueforti*. *PLOS ONE*, 1-10. DOI:10.1371/journal.pone.0171387.

Schepers, A. W., Thibault, J., & Lacroix, C. (2002). *Lactobacillus helveticus* growth and lactic acid production during pH controlled batch cultures in whey permeate/yeast extract medium. Part I. multiple factor kinetic analysis. *Enzyme and Microbial Technology, 30*, 176-186.

Singh, T. K., Drake, M. A., & Cadwallader, K. R. (2003). Flavour of Cheddar Cheese: a chemical and sensory perspective. *Comprehensive Reviews in Food Science and Food Safety,* 2, 139-162.

Vítová, E., Loupancová, B., Zemanová, J., Štoudková, H., Březina, P., & Babák, L. (2006). Solid-phase microextraction for analysis of mould cheese aroma. *Czech Journal of Food Sciences, 24*, 268-274.

Whitley, E. (2002). *The microflora of blue Stilton cheese. Thesis*. The University of Nottingham.

Wouters, J. T. M., Ayad, E. H. E., Hugenholtz, J., & Smit, G. (2002). Microbes from raw milk for fermented dairy products. *International Dairy Journal, 12*, 91-109.

ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

Table 1

Viable counts, lactic and acetic acid production, and pH changes in MRS broth inoculated with *Lactobacillus plantarum* isolates from Stilton cheese and incubated at 30°C. Values are means of three independent analyses ± standard errors of the means.

Time (h)	R2 (crust)		R6 (crust)		B30 (veins)		R25 (veins)		R40 (core)		W8 (core)	
	Log_{10} CFU/mL	pH	Log_{10} CFU/mL	pH	Log_{10} CFU/mL	pH	Log_{10} CFU/mL	pH	Log_{10} CFU/mL	pH	Log_{10} CFU/mL	pH
0	3.69±0.06^{f}	5.79±0.00^{a}	3.69±0.54^{d}	5.79±0.00^{a}	3.58±0.09^{e}	5.79±0.00^{a}	3.18±0.18^{f}	5.79±0.00^{a}	3.31±0.18^{d}	5.79±0.00^{a}	3.89±0.08^{e}	5.79±0.00^{a}
3	4.17±0.15^{e}	5.69±0.06^{b}	3.77±0.29^{d}	5.53±0.06^{b}	4.08±0.12^{d}	5.46±0.03^{c}	3.74±0.11^{e}	5.43±0.03^{c}	3.74±0.20^{d}	5.46±0.01^{c}	4.46±0.08^{d}	5.41±0.01^{c}
6	4.71±0.10^{d}	5.57±0.01^{c}	4.36±0.21^{d}	5.59±0.01^{b}	4.71±0.17^{c}	5.58±0.04^{b}	4.56±0.04^{d}	5.65±0.01^{b}	4.56±0.24^{c}	5.57±0.05^{b}	5.32±0.05^{c}	5.56±0.09^{b}
9	5.24±0.21^{c}	5.56±0.01^{c}	4.97±0.29^{c}	5.72±0.06^{a}	5.43±0.19^{b}	5.60±0.02^{b}	4.97±0.11^{c}	5.79±0.06^{a}	5.00±0.10^{c}	5.57±0.02^{b}	5.85±0.08^{b}	5.61±0.05^{b}
24	8.32±0.22^{b}	5.01±0.01^{d}	7.63±0.46^{b}	5.44±0.08^{c}	8.43±0.11^{a}	4.64±0.08^{d}	8.10±0.12^{b}	5.04±0.02^{d}	7.90±0.15^{b}	5.25±0.08^{d}	9.03±0.15^{a}	4.26±0.05^{d}
48	8.81±0.10^{a}	4.07±0.05^{e}	8.96±0.15^{a}	4.09±0.03^{d}	8.52±0.09^{a}	3.92±0.01^{e}	9.01±0.10^{a}	3.93±0.06^{e}	8.77±0.11^{a}	3.98±0.04^{e}	9.13±0.12^{a}	3.76±0.01^{e}
	Lactic acid (g/L)	Acetic acid (g/L)	Lactic acid (g/L)	Acetic acid (g/L)	Lactic acid (g/L)	Acetic acid (g/L)	Lactic acid (g/L)	Acetic acid (g/L)	Lactic acid (g/L)	Acetic acid (g/L)	Lactic acid (g/L)	Acetic acid (g/L)
0	0.00±0.00^{c}	0.00±0.00^{b}	0.00±0.00^{c}	0.00±0.00^{b}	0.00±0.00^{c}	0.00±0.00^{b}	0.00±0.00^{c}	0.00±0.00^{b}	0.00±0.00^{c}	0.00±0.00^{b}	0.00±0.00^{c}	0.00±0.00^{b}
3	0.02±0.07^{c}	0.15±0.46^{b}	0.07±0.05^{c}	0.30±0.22^{b}	0.09±0.03^{c}	0.26±0.17^{b}	0.02±0.10^{c}	0.35±0.27^{b}	0.07±0.03^{c}	0.33±0.26^{a,b}	0.07±0.03^{c}	0.27±0.26^{b}
6	0.03±0.09^{c}	0.15±0.38^{b}	0.09±0.02^{c}	0.47±0.35^{b}	0.01±0.07^{c}	0.31±0.33^{b}	0.05±0.01^{c}	0.24±0.26^{b}	0.04±0.03^{c}	0.15±0.17^{a,b}	0.04±0.03^{c}	0.16±0.13^{b}
9	0.02±0.03^{c}	0.15±0.28^{b}	0.05±0.03^{c}	0.24±0.25^{b}	0.08±0.02^{c}	0.37±0.34^{b}	0.04±0.03^{c}	0.23±0.22^{b}	0.06±0.03^{c}	0.26±0.34^{a,b}	0.02±0.05^{c}	0.07±0.15^{b}
24	2.14±0.13^{b}	0.29±0.23^{b}	0.75±0.26^{b}	0.18±0.23^{b}	4.36±0.43^{b}	0.31±0.21^{a,b}	1.84±0.45^{b}	0.55±0.22^{a,b}	1.14±0.20^{b}	0.28±0.21^{a,b}	7.99±0.92^{b}	0.39±0.27^{b}

ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

48	13.63±0.82[a]	1.75±0.46[a]	13.91±0.33[a]	1.37±0.46[a]	14.06±0.78[a]	0.99±0.34[a]	15.19±0.96[a]	1.06±0.08[a]	13.64±0.42[a]	0.71±0.16[a]	21.40±1.45[a]	1.34±0.13[a]

Values in columns with same superscript letters are not significantly different (p>0.05)

Table 2

Average SPME GC-MS signal intensities (and standard deviations, SD) for compounds detected from headspace samples of fermented milk inoculated with single and mixed cultures of *Lactobacillus plantarum* isolates and different strains of *Lactococcus lactis*. The samples were incubated for 48 h at 30°C. B30, R2 and W8: isolates of *Lb. plantarum* from the blue veins, outer crust and white core, respectively. LcA: acid producing *Lc. lactis*, LcNA: non-acid producing *Lc. lactis* NCIMB 9918.

		Single strain cultures					Mixed strain cultures						Milk (control)
		Lb. plantarum			*Lc. lactis*		*Lb. plantarum* + LcA			*Lb. plantarum* + LcNA			
Compound		B30	R2	W8	LcA	LcNA	B30-LcA	R2-LcA	W8-LcA	B30-LcNA	R2-LcNA	W8-LcNA	
Alcohols													
Ethyl alcohol (ethanol)	Mean	0.16	0.28	0.20	0.26	0.37	0.39	0.31	0.58	0.38	0.49	0.63	0.16
	SD	0.03	0.21	0.05	0.06	0.04	0.09	0.03	0.38	0.20	0.64	0.40	0.12
2-methyl propanol	Mean	0.06	0.09	0.06	1.65	0.09	6.13	1.78	1.86	0.08	0.10	0.11	0.08
	SD	0.02	0.06	0.01	0.24	0.06	0.24	0.21	0.35	0.01	0.03	0.04	0.04
3-methyl butanol	Mean	0.01	0.03	0.01	5.26	0.07	62.08	4.83	4.91	0.03	0.02	0.02	0.02
	SD	0.01	0.03	0.01	1.56	0.10	3.82	0.41	0.66	0.02	0.01	0.01	0.01
2-methyl butanol	Mean	0.05	0.07	0.06	5.17	0.10	16.40	4.93	5.27	0.07	0.06	0.06	0.08
	SD	0.01	0.04	0.03	0.90	0.06	0.47	1.23	1.01	0.04	0.04	0.02	0.06
Hexanol	Mean	0.01	0.02	0.02	0.05	0.02	0.06	0.03	0.05	0.01	0.01	0.02	0.01
	SD	0.01	0.02	0.00	0.02	0.01	0.01	0.00	0.01	0.00	0.01	0.02	0.01
Carboxylic acids													
Acetic acid	Mean	0.00	0.01	0.00	0.00	0.01	0.05	0.00	0.00	0.00	0.00	0.00	0.01

ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

	SD	0.00	0.01	0.00	0.00	0.00	0.03	0.00	0.00	0.00	0.00	0.00	0.01
Butanoic acid	Mean	0.00	0.01	0.00	0.06	0.01	0.17	0.00	0.00	0.00	0.00	0.01	0.02
	SD	0.00	0.01	0.00	0.10	0.00	0.06	0.00	0.00	0.00	0.00	0.01	0.04
Hexanoic acid	Mean	0.00	0.00	0.00	0.18	0.00	0.04	0.00	0.01	0.00	0.00	0.00	0.02
	SD	0.00	0.00	0.00	0.30	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.04
Ketones													
2-pentanone	Mean	0.41	0.53	0.40	0.41	0.52	0.39	0.40	0.37	0.45	0.49	0.55	0.58
	SD	0.03	0.22	0.04	0.09	0.20	0.03	0.03	0.03	0.12	0.15	0.31	0.30
Acetoin	Mean	0.15	0.34	0.15	0.38	2.61	0.85	0.40	0.30	2.26	2.62	2.91	0.16
	SD	0.01	0.16	0.07	0.11	1.13	0.07	0.18	0.05	0.83	1.26	1.74	0.10
2-hexanone	Mean	0.04	0.06	0.04	0.04	0.06	0.05	0.04	0.05	0.05	0.07	0.07	0.08
	SD	0.01	0.03	0.01	0.02	0.04	0.01	0.01	0.01	0.02	0.04	0.07	0.04
2-hydroxy 3-pentanone	Mean	0.02	0.03	0.02	0.09	0.06	0.13	0.07	0.07	0.04	0.04	0.04	0.08
	SD	0.01	0.02	0.02	0.05	0.03	0.02	0.03	0.01	0.01	0.01	0.03	0.06
2-heptanone	Mean	2.57	3.70	2.57	2.59	3.79	2.76	2.20	2.15	2.85	3.55	4.00	4.12
	SD	0.25	1.81	0.54	0.88	1.86	0.24	0.21	0.16	1.01	1.45	2.99	2.36
2-nonanone	Mean	0.18	0.30	0.27	0.20	0.33	0.20	0.17	0.16	0.26	0.29	0.40	0.40
	SD	0.04	0.24	0.17	0.11	0.25	0.03	0.02	0.02	0.16	0.18	0.43	0.30
Aldehydes													
2-methyl propanal	Mean	0.01	0.01	ND	1.02	0.01	0.97	1.08	0.99	0.01	0.02	0.02	0.02
	SD	0.01	0.01	ND	0.07	0.00	0.19	0.10	0.17	0.01	0.01	0.01	0.01
3-methyl butanal	Mean	0.02	0.08	0.06	1.65	0.05	47.42	2.30	1.60	0.05	0.06	0.07	0.10
	SD	0.01	0.01	0.00	0.09	0.03	2.68	0.94	0.30	0.03	0.06	0.05	0.10
2-methyl butanal	Mean	0.01	0.01	0.00	2.38	0.01	13.94	2.40	2.08	0.01	0.01	0.01	0.04
	SD	0.00	0.01	0.00	0.19	0.01	1.33	0.35	0.23	0.01	0.01	0.00	0.02
Esters													
Butanoic acid 2-methyl ester	Mean	0.01	0.01	0.00	0.09	0.01	0.33	0.11	0.12	0.00	0.01	0.01	0.01
	SD	0.00	0.02	0.00	0.02	0.01	0.03	0.02	0.03	0.00	0.00	0.01	0.01
Butanoic acid ethyl ester	Mean	0.01	0.03	0.02	0.02	0.03	0.02	0.01	0.02	0.02	0.04	0.04	0.06
	SD	0.01	0.02	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.02	0.03	0.06
Butanol 3-methyl acetate	Mean	0.00	0.00	0.00	0.01	0.01	0.08	0.00	0.01	0.00	0.00	0.01	0.01
	SD	0.00	0.00	0.00	0.00	0.01	0.02	0.00	0.00	0.00	0.00	0.00	0.01
Propanoic acid methyl ester	Mean	0.00	0.03	0.00	0.01	0.03	0.00	0.01	0.00	0.01	0.08	0.03	0.12
	SD	0.00	0.05	0.00	0.00	0.04	0.00	0.01	0.00	0.01	0.08	0.04	0.18

ACCEPTED MANUSCRIPT

Values are relative to the signal intensity observed when the headspace above a 5 µg/l 2-nonanone solution was sampled into GC-MS. The values are means of three independent replicates.

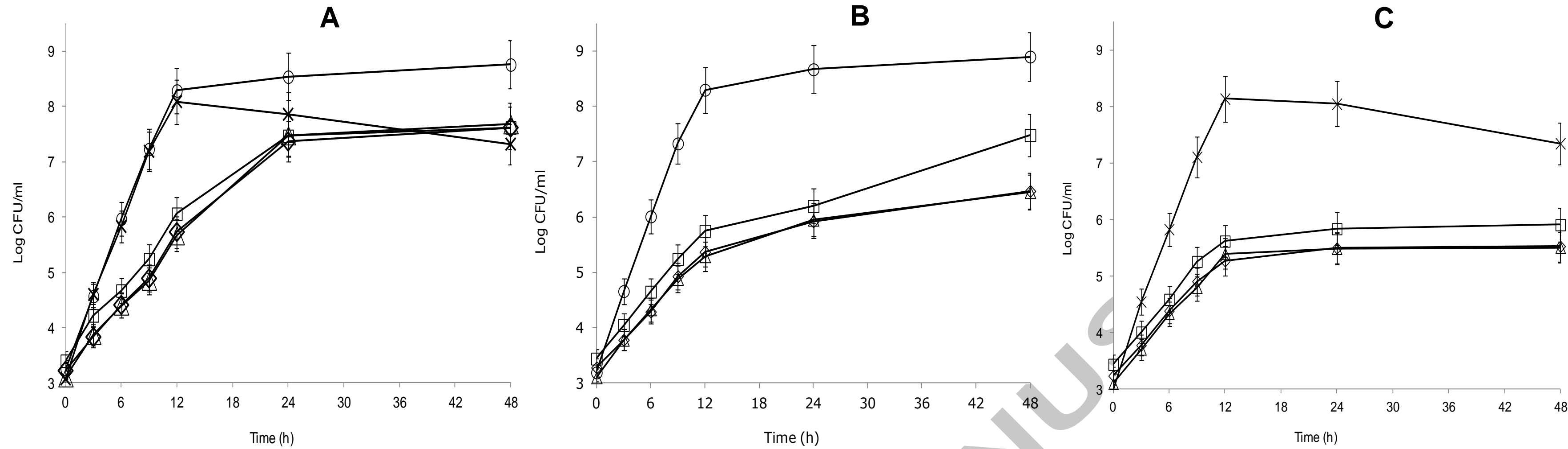
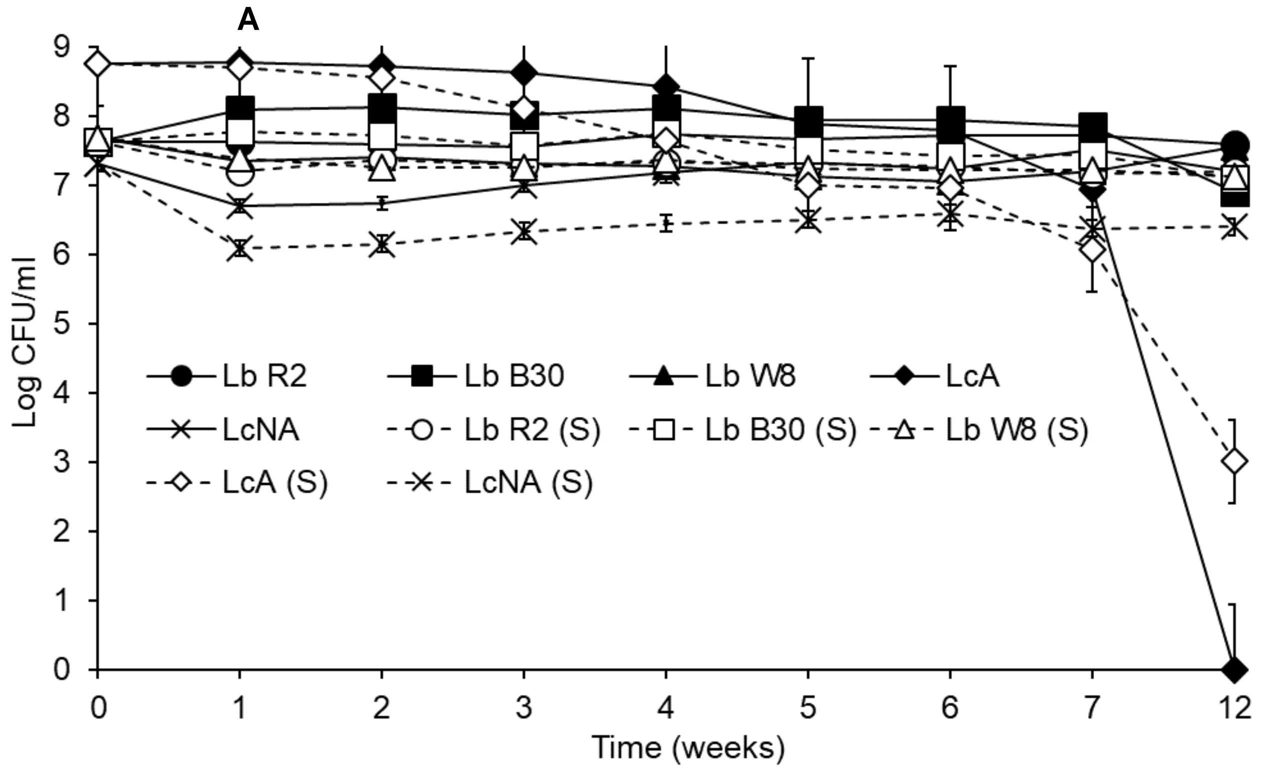
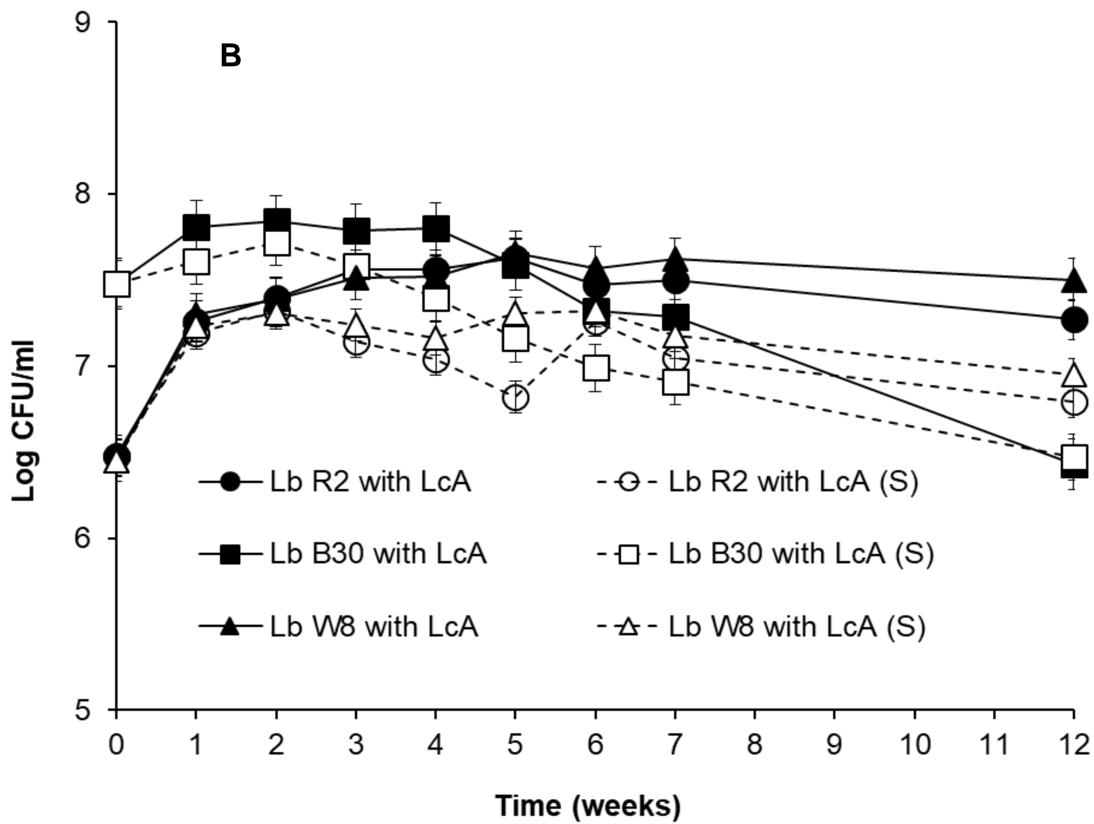


Fig. 1. Growth of *Lactobacillus plantarum* isolates in milk at 30°C. (**A**) single strain cultures, (**B**) isolates cultured with acid producing *Lactococcus lactis*; (**C**) isolates cultured with non-acid producing *Lc. lactis*. *Lb. plantarum* isolates: (◊) R2, outer crust; (□) B30, blue veins; and (∆) W8, white core. (o) Acid producing *Lc. lactis*; (X) non-acid producing *Lc. lactis*. Points are means of three independent experiments and error bars are ± standard errors of the means.

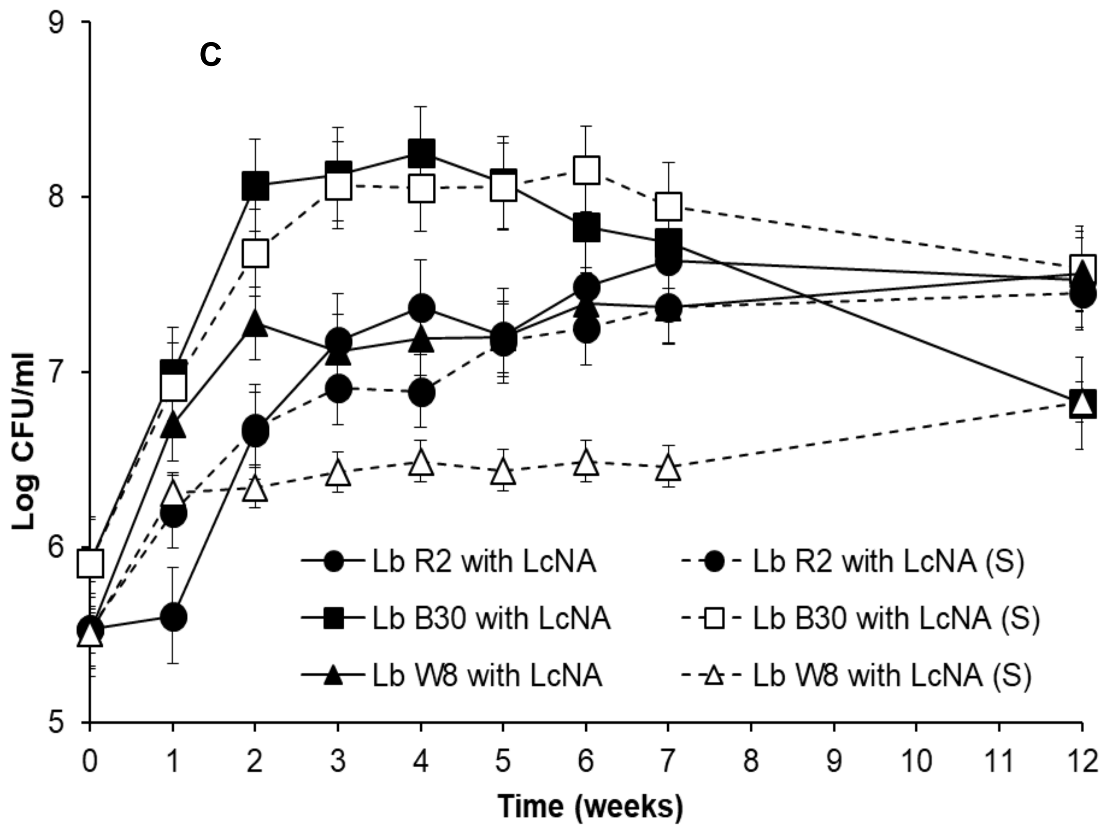
ACCEPTED MANUSCRIPT

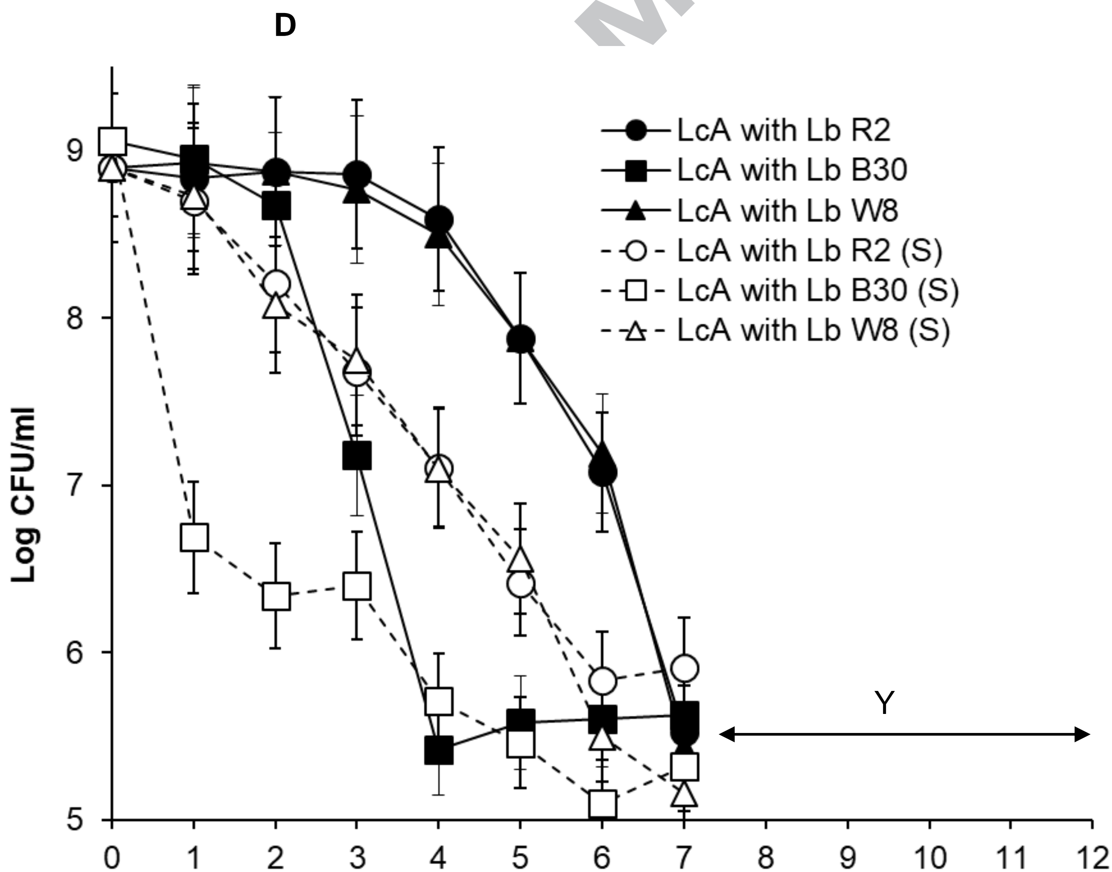
ACCEPTED MANUSCRIPT





ACCEPTED MANUSCRIPT





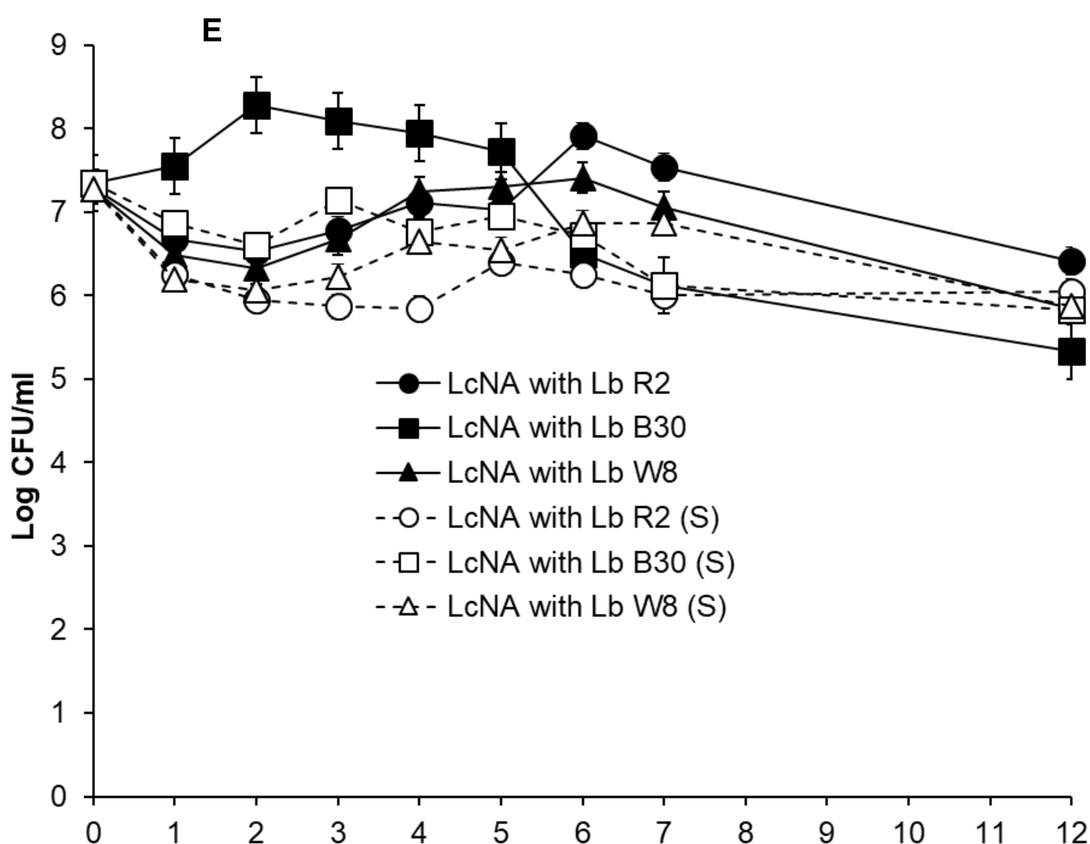
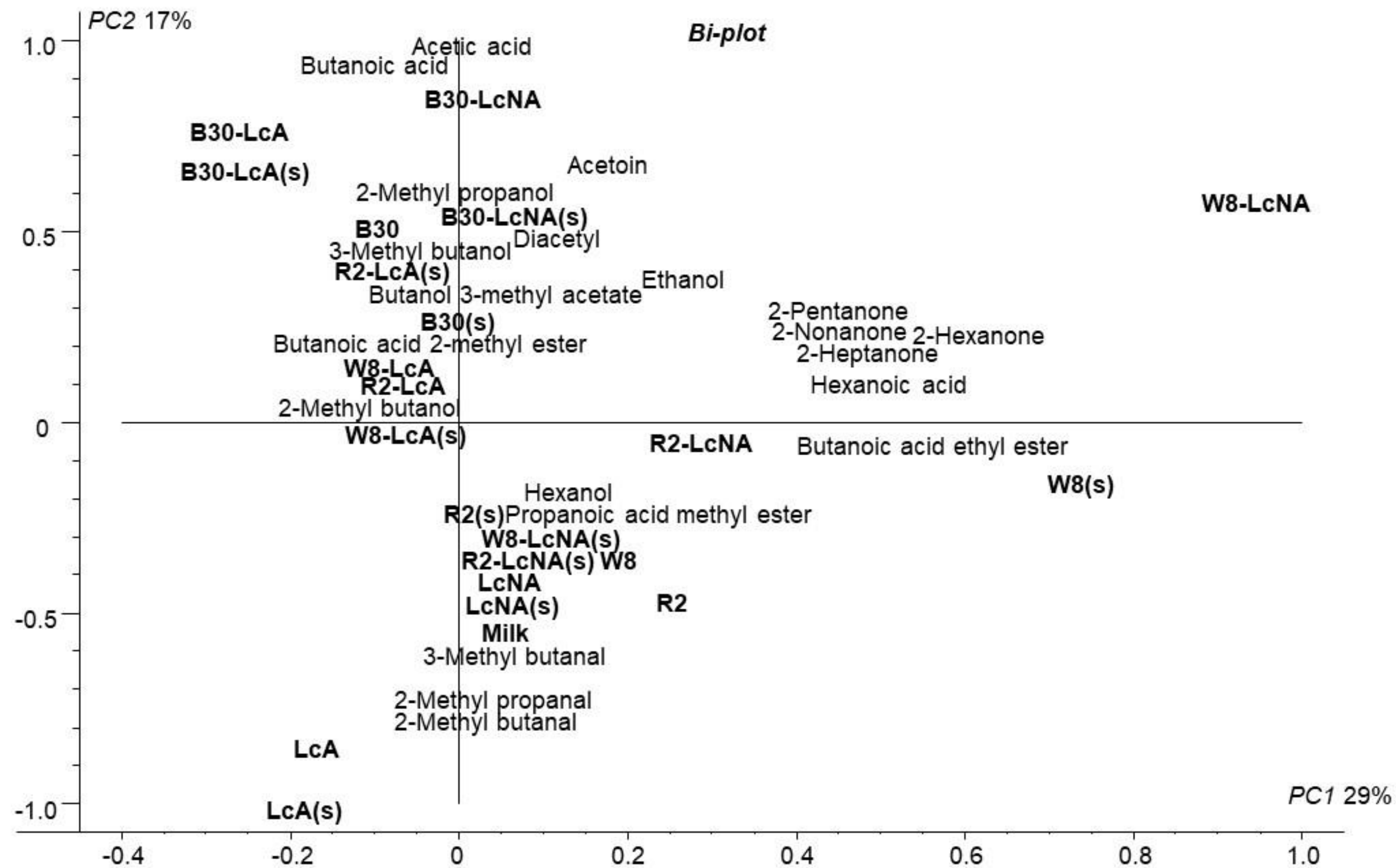


Fig. 2. Growth in milk at 18°C in the presence (dotted lines) and absence of salt (solid lines). (**A**) Single strain cultures, (**B**) *Lactobacillus* isolates co-cultured with LcA, **(C)** *Lactobacillus* isolates co-cultured with LcNA, **(D)** LcA co-cultured with *Lactobacillus* isolates, **(E)** LcNA co-cultured with *Lactobacillus* isolates. *Lb. plantarum* isolates: (o) R2, outer crust; (□) B30, blue veins; and (Δ) W8, white core. *Lactococcus lactis* strains: (◊) Acid producer, LcA; (X) Non-acid producer, LcNA. Points are means of three independent experiments and error bars are ± standard errors of the means.

ACCEPTED

ACCEPTED MANUSCRIPT



ACCEPTED

ACCEPTED MANUSCRIPT

Fig. 3. PCA plot of the volatile compounds detected using SPME GC-MS analysis of headspace samples of milk inoculated with *Lactobacillus plantarum* in single culture or co-culture with *Lactococcus lactis*. Strain numbering: *Lb. plantarum* R2 (outer crust), B30 (blue veins), W8 (white core), acid producing *Lc. lactis* (LcA) and non-acid producing *Lc. lactis* (LcNA). The cultures were incubated for 48 h at 30°C and then for seven weeks at 18°C in salted (s) and unsalted milk. Means for scores and loadings of three independent replicates of each sample are shown.

ACCEPTED MANUSCRIPT