Effect of Starter Culture on Accumulation of Taste Active Amino Acids, Free Fatty Acids, and on Survival of Pathogenic *Escherichia coli* in Dry Fermented Beef Sausages

by

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Abstract

The accumulation of taste active compounds during ripening determines the taste of fermented meats; however, the contribution of defined starter cultures to glutamate during sausage ripening remains unknown. This study investigated the role of lactic acid bacteria and *Staphylococcus carnosus* on the accumulation of free amino acids during dry sausage fermentation. A sausage model system was developed to ensure control of sausage microbiota throughout ripening. Sausages were produced at the laboratory-scale with defined starter cultures; aseptic controls were fermented without culture addition. *Lactobacillus sakei* FUA3009, *Lactobacillus plantarum*, *Pediococcus pentosaceus* FUA3071, *Pediococcus acidilactici* were used as single cultures; *Staphylococcus carnosus* with *L. sakei* FUA3549 or *P. pentosaceus* FUA3550 were used as cocktails. The cell counts in aseptic control sausages remained <1 log CFU g^{-1} throughout 20 d of ripening. The water activity and pH of model sausages were comparable to commercial products. The use of the model system demonstrated that bacterial enzymes influenced the release of free amino acids, even during the initial fermentation stage. Ripening time and starter cultures are important factors determining the accumulation of free amino acids. By controlling the sausage microbiota with clean meat and amino acid decarboxylase negative strains, biogenic amines remained below the detection limit in all products. The increase of total free fatty acids at the end of ripening stage was primarily catalyzed by the endogenous lipase, and the unsaturated fatty acids were further utilized by starter cultures. The sausage model system was also used for a challenge trial with a cocktail of pathogenic *E. coli* strains; cell counts of pathogenic *E. coli* were reduced by less than 1 Log CFU g^{-1} during ripening. The sausage model for control of ripening microbiota will facilitate further studies on the impact of defined cultures on the safety and quality of fermented meats.

Preface

This thesis is an original work by Kaixing Tang.

This thesis with the exception of fatty acids analysis has been poster presented at 12th International Symposium on Lactic Acid Bacteria and submitted as Kai Xing Tang, Tiange Shi, Michael Gänzle. Effect of starter culture on taste active amino acids and survival of pathogenic *Escherichia coli* in dry fermented beef sausages. International Journal of Food Microbiology. Tiange Shi developed the sausage model system for her undergraduate research project. Kaixing Tang modified the model system, conducted all experimental works, and drafted the manuscript. Dr. Michael Gänzle, Dr. Yuanyao Chen, and Weilan Wang contributed to the experimental design primer design, and statistical analysis respectively. Dr. Michael Gänzle and Tiange Shi helped with the revision of the manuscript.

LC-MS method development of identification and quantification of non-hydroxy and hydroxy fatty acids in fermented sausages was performed by Nuanyi Liang. Kaixing Tang conducted the sausage fermentation and fatty acids extraction. Nuanyi Liang developed the analytical method to quantify the fatty acids and hydroxyl fatty acids from dry sausages.

Dedication

To my parents and family,

Thank you for your love, encouragement, and support.

Acknowledgements

My passion for research started from the first research project I did on type I sourdough fermentation in Lab 2-50 during my undergraduate study. During the research project, supervisor Dr. Michael Gänzle and mentor Dr. Cindy Zhao provided me with tremendous support, and with their encouragement and inspiration I started my journey to Master's degree in Food Science and Technology.

I am grateful for having the best supervisor for the past two years; Dr. Michael Gänzle, thank you for recognizing my potential and placing the confidence in me. You provided me with the opportunities to mentor students, attend conference, and share knowledge among peers. Your encouragement and support made my thesis research an interesting and memorable experience.

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I would especially like to thank my parents for always being my biggest support anytime. Recognizing my potential, supporting my life choices, providing help and guardiancy and encouraging me to continue to strive toward excellence.

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List of Abbreviations

ADI	Arginine-deiminase
a_W	Water activity
CFU	Colony Forming Units
CT-SMAC	Sorbitol MacConkey Agar with cefixime and tellurite
d	Day
DM	Dry matter
DNA	Deoxyribonucleic acid
EHEC	Enterohemorrhagic *Escherichia coli*
GABA	γ-amino butyrate
GAD	Glutamate decarboxylase
GDL	Glucono delta-lactone
h	Hours
HPLC	High-performance liquid chromatography
LB	Luria-Bertani
LC-ESI-MS/MS	Liquid chromatography coupled with negative-ion electrospray ionization-tandem mass spectrometry
Log	Logarithmic
min	Minutes
MRS	De Man, Rogosa and Sharpe
OH	Hydroxy
PCA	principle component analysis
PCR	Polymerase Chain Reaction
s	Seconds
TSA	Tryptic Soy Agar
wt	Weight

1. Introduction

1.1 Overview

Sausage fermentation preserves meat without thermal processing; fermented sausages are also characterized by a characteristic flavor (Lachowicz et al., 2012). The variation of ingredients, caliber, fermentation conditions and fermentation microbiota results in a large diversity of products. Dry fermented sausages are found in different parts of the world and some of the typical Mediterranean dry cured sausages are Italian salami, Spanish chorizo, and French *saucisson* (Lachowicz et al., 2012). Fermented sausages can be made from a variety of meat including pork, beef, poultry and mutton, and mixed with curing salt, sugar, spices, and starter cultures (Lachowicz et al., 2012; Vignolo et al., 2010). Many traditional fermented sausages are produced based on spontaneous fermentation process, and the indigenous meat lactic acid bacteria commonly found in traditional cured sausages are *Lactobacillus sakei*, *Lactobacillus plantarum*, and *Lactobacillus curvatus* (Lachowicz et al., 2012; Vignolo et al., 2010). Safety and sensory properties of fermented meats are dependent on species- or strain specific metabolic traits of fermentation organisms. Past studies with sausage model systems did not provide an adequate control of fermentation microbiota in fermented products (Fadda et al., 2002; López et al., 2015a; López et al., 2015b). This study therefore aimed to develop an aseptic sausage model system to determine the impact of defined fermentation organisms on the safety and quality of fermented sausages.

1.2 Use of starter cultures in commercial sausage production

The use of commercial starter culture for sausage fermentation is necessary for the modern sausage production, in terms of process and product standardization, and the selection of sausage starter culture depends on processing conditions and sausage types (Lachowicz et al., 2012; Vignolo et al., 2010). Dry cured sausages are fermented by lactobacilli including

Pediococcus spp., and *Micrococcaceae* or staphylococci that are present as natural fermentation microbiota, or added as starter cultures (Lachowicz et al., 2012; Vignolo et al., 2010). Fungi and yeasts (ex. *Penicillium camemberti* and *Debaryomyces hansenii*) are used for surface ripened sausages (Hammes and Hertel, 1998). Sausage batter is stuffed in natural or artificial casings, and subjected to fermentation and a minimum 28 days ripening process (Lachowicz et al., 2012; Vignolo et al., 2010). Sausage starter cultures are important for both quality and safety of the final product. Acidification by lactic acid bacteria coagulates meat proteins and reduces microbial risks in fermented sausage (Lachowicz et al., 2012; Ordóñez et al., 1999; Vignolo et al., 2010). Micrococci and staphylococci have catalase and nitrate reductase activities that are important for sausage colour formation and stabilization (Hammes and Hertel, 1998).

Canadians are increasingly demanding ready-to-eat products over past few years, and the sales of processed meat products including bacon, sausage, and luncheon meat have grown over the years (Fernando, 2017). From 2010 to 2015, the processed sausage sales in Alberta increased from 4 million kg to 5.5 million kg, with an average price increase of $2 per kg annually (Fernando, 2017). Driven by the consumer's demand in convenience and growing immigration population, the demand of new and unique flavour and the processed meat market is expected to grow in the future (Fernando, 2017). In order to assist the increasing market demand on high quality and tasty dry cured sausages, this thesis project investigated dry cured sausage fermentations, which can provide guidance to the industry in terms of the flavour development and safety controls.

1.3 Flavour development in dry fermented sausages

During dry sausage fermentation, biochemical changes including proteolysis and lipolysis contribute to flavour and texture of the products by generating taste or flavor active peptides, amino acids, aldehydes, and organic acids (Lachowicz et al., 2012; Molly et al., 1997; Ordóñez

et al., 1999; Toldrá, 1998; Fig. 1). The endogenous meat protease cathepsin (B, D, H, and L) mediates initial protein degradation. *Lactobacillus* spp. and *Pediococcus* spp. additionally release amino acids by intracellular peptidases (Ordóñez et al., 1999). Amino acids and short peptides produced by sarcoplasmic and myofibrillar protein hydrolysis contribute directly to meat flavour, or are precursors for the microbial synthesis of flavor compounds. *Staphylococcus xylosus* and *Staphylococcus carnosus* metabolize the branched-chain amino acids leucine, isoleucine and valine to flavor-active aldehydes, alcohols, and methyl ketones, which contributed to the typical perceived cured salami flavor (Lachowicz et al., 2012; Stahnke et al., 2002; Vignolo et al., 2010). Lactobacilli also convert amino acids to flavor volatiles, predominantly organic acids, which contributes to the mild acidity of the product (Sinz and Schwab, 2012).

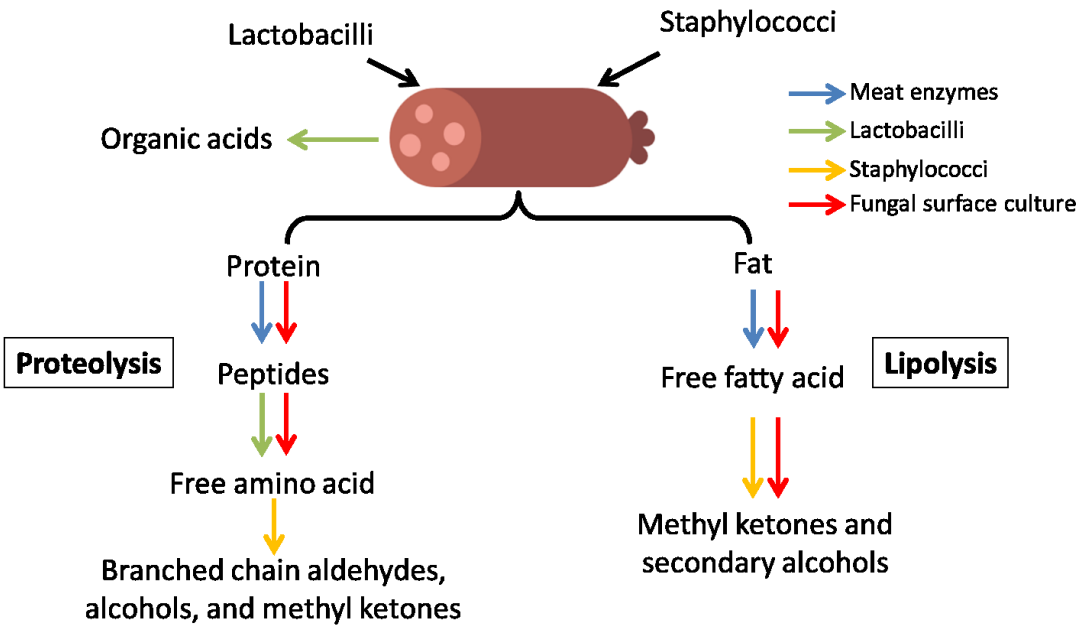

Figure 1. Flavour generation in dry cured sausages. The drawing is based on the information provided by Toldrá (1998).

Accumulation of glutamate during food fermentation depends on proteolysis, and on strain specific conversion of glutamine by glutaminase has been investigated in sourdough and cheese (Teixeira et al., 2014; Toelstede and Hofmann, 2008; Vermeulen et al., 2007; Zhao et al.,

2015; Zhao et al., 2016; Zheng et al., 2015). The contribution of the strain specific conversion of glutamine to accumulation of taste active glutamate in dry cured sausages, however, requires control of fermentation microbiota and has to date not been investigated. Most studies on dry fermented sausage studies were performed at the pilot scale without comparison to aseptic controls (Ordóñez et al., 1999). Therefore, the current research aimed to develop a sausage model system which allows the use of aseptic control, and then investigate the role of starter cultures to the accumulation of taste active amino acids.

Micrococcaeae and staphylococci have high lipolytic activity, but the overall lipolysis is predominately catalyzed by the endogenous lipase during sausage ripening (Molly et al., 1997; Ordóñez et al., 1999; Zuber and Horvat, 2007). Bacterial enzymes play an important role on oxidation of free fatty acids to volatile compounds, and methyl ketones are intermediates derived from the incomplete β-oxidation by staphylococci (Leroy et al., 2006). Additionally, nitrate reductase and catalase positive staphylococci control off-flavour formation by preventing unsaturated fatty acid oxidation (Montel et al., 1998). Some lactobacilli convert linoleic acid to hydroxy fatty acids, which demonstrated antifungal activity in fermented sourdough bread (Black et al., 2013). Studies on lipid metabolism in sausage fermentation primarily focused on the contribution to the flavour profile, and the production of hydroxy fatty acids has not yet been investigated (Casaburi et al., 2008; Hammes and Knauf, 1994; Leroy et al., 2006; Montel et al., 1998; Toldrá, 1998). Therefore, this study investigated the contribution of defined starter culture to free fatty acids metabolism in dry cured sausages.

1.4 Safety of dry fermented sausages

Biogenic amines are toxic substances, which are mainly generated by microbial decarboxylation of amino acids, and tyramine and putrescine are common biogenic amines found in dry fermented sausage (Suzzi and Gardini, 2003). The toxic levels of biogenic amines are not

precisely known, but nitrosamine, which is potentially carcinogenic, can be formed by biogenic amines in nitrite/nitrate cured meat products (Bover-Cid et al., 1999). The biogenic amine in fermented sausages is highly variable; their accumulation is controlled by using competitive and decarboxylase-negative starter cultures, clean raw materials, and suitable processing conditions (Suzzi and Gardini, 2003).

The safety of fermented sausages is mainly dependent on rapid acidification. Additionally, some lactic acid bacteria produce bacteriocin, which is used for sausage fermentation to reduce hygienic risks (Hammes and Knauf, 1994). Bacteriocin producing starter cultures allow control of *Listeria monocytogenes* in fermented sausages but are not effective against pathogenic *Escherichia coli* O157:H7 (Lahti et al., 2001). The reduction of cell counts of *E. coli* O157 during sausage fermentation and ripening ranges from 1 to 4 log CFU g^{-1} (Holck et al., 2011). Survival of pathogenic *E. coli* O157:H7 during the dry sausage manufacture caused several outbreaks and recalls, thus food regulatory agencies in both Canada and the U.S. require the manufacturer to validate the process which is capable of reducing *E. coli* O157:H7 for at least 5 log CFU g^{-1} (Muthukumarasamy and Holley, 2007). Although manufacturers are taking precautions to ensure the product safety, a number of cases of recalls associated with shiga-toxin producing *E. coil* in dry cured salami every year. The most recent recall on shiga-toxin producing *E. coli* in salami was issued in February 2018 in Austria (European Commission Food and Safety Alerts database), and the massive recalls were issued to withdraw the products from the both domestic and international markets.

Post processing strategies including smoking and heating are essential and commonly used to reduce *E. coli* in dry cured sausages (Holck et al., 2011). Most of the studies used only selective media to examine the survival of *E. coli*, which overestimated the cell reduction,

because sublethal injured cells can recover under nutrient conditions (Table 1). *E. coli* O157:H7 has long been considered as prototype for enterohemorrhagic *E. coli* (EHEC) (Holck et al., 2011). Although other serotypes of EHEC (O26, O103, O45, O111, O121, and O145) later were found as significant contributors to foodborne illness (Balamurugan et al., 2017), most studies on EHEC survival in dry sausages production used single strain *E. coli* O157:H7 for the investigation (Cordeiro et al., 2013; Ducic et al., 2016; Luciano et al., 2011). Reduction of EHEC was strain dependent under dry fermented sausage production and post processing conditions (Rode et al., 2012a). Therefore, this study aimed to examine the survival of pathogenic *E. coli* by using the strain cocktail and both selective and non-selective media for cell counts.

Table 1. Studies on reduction of *E. coli* in fermented sausages.

Reference	Time (d)	*E. coli* single strain/cocktail	$\log_{10}$ reduction	Media used for cell count[a]	*E. coli* reduction strategies
(Luciano et al., 2011)	38	Single strain	3.6 to >5	CT-SMAC	Mustard addition (6% wt/wt)
(Rode et al., 2012a)	23	Single strain	1 to 5	TSA	Heat treatment, extended cold storage, and freezing/thawing
(Cordeiro et al., 2013)	35	Single strain	2 to >5	CT-SMAC	Mustard addition (4% wt/wt)
(Heir et al., 2013)	23	Cocktail	3 to >5	TSA with rifampicin	High salt and glucose recipe, heat treatment, freezing/thawing, and extended storage
(Ducic et al., 2016)	15	Single strain	3 to >5	CT-SMAC	heat treatment and extended cold storage
(McLeod et al., 2016)	23	Single strain	1 to 4	TSA with rifampicin	High salt and sugar recipe, high fermentation temperature, and extended storage

[a] Media abbreviation: CT-SMAC: Sorbitol MacConkey Agar with cefixime and tellurite; TSA: Tryptic Soy Agar

1.5 Hypotheses

This thesis aimed to test the following hypotheses: 1) generation of fermentation metabolites, particularly glutamate, in dry cured sausages are strain specific; 2) acidification and drying during sausage fermentation and ripening are insufficient to reduce pathogenic *E. coli* strain cocktail by 5 log CFU g^{-1}.

2. Material and Methods

2.1 Strains and growth conditions

L. sakei FUA3009 and FUA3549, *Lactobacillus plantarum* FUA3073, *Pediococcus acidilactici* FUA3072, and *Pediococcus pentosaceus* FUA 3071 and FUA3550 were cultivated in De Man, Rogosa and Sharpe (MRS) media at 30°C anaerobically for 16 h. *S. carnosus* FUA2133 was routinely grown on MRS media at 37°C aerobically for 16 h. *L. sakei* FUA 3009 and FUA3549 and *S. carnosus* FUA2133 are isolates from commercial meat starter cultures; other strains are isolates from retail meat. A five strain cocktail of pathogenic *E. coli* was prepared as described (Garcia-Hernandez et al., 2015) to contain Shiga toxin producing *E. coli* O26:H11, O121:H19, O145:NM 03-6430, O157:H7, O145:NM and the enteropathogenic *E. coli* O145:NM PARC 499. *E. coli* were routinely grown in Luria-Bertani (LB) broth and incubated aerobically at 37°C with 200 rpm agitation.

2.2 Strain identity and confirmation *gadB* gene in *L. plantarum*

The identity of starter cultures was confirmed by sequencing of genes coding for 16S rRNA. DNA was isolated from 1 mL overnight culture using DNeasy blood and tissue kit according to the instruction provided by the manufacturer (Qiagen, Mississauga, ON, Canada). Polymerase chain reaction (PCR) amplified the 16S rRNA genes of each starter culture. PCR reaction was performed in a volume of 25µL containing 1 µL template DNA, 17.5 µL autoclaved water, 2.5 µL 10× buffer, 0.75 µL 50 mM MgCl, 0.5 µL 10mM dNTP, 1.25 µL 10µM 27F

primer (AGAGTTTGATCMTGGCTCAG), 1.25 µL 10 µM 1492R primer (TACGGYTACCTTGTTACGACTT), and 0.25 µL *Taq* DNA polymerase. The PCR conditions were as follows: initial denaturation at 94°C for 5 min, 30 cycles of denaturation at 94°C for 45s, annealing at 58°C for 45 s, extension at 72°C for 90 s. Final extension was at 72°C for 7 min, and PCR product was held at 4°C. PCR product was sequenced by service of Macrogen (Rockville, USA). The *gadB* gene in *L. plantarum* was amplified by PCR reaction using primers *gadB* F (CTAAGTATCGGTTACCAAAGCATTC) and *gadB* R (GTGTGTGAATCCGTATTTCTTAGG) under the same condition as described with annealing temperature 54°C for 1 min. Amplification was verified by agarose gel electrophoresis.

2.3 Preparation of inoculum for meat fermentations.

L. sakei FUA3009, *L. plantarum* FUA3073, *P. pentosaceus* FUA3071, and *P. acidilactici* FUA3072 were each prepared as a single strain. *S. carnosus* with *L. sakei* FUA3549 or *P. pentosaceus* FUA3550 were used as cocktails. Strains were subcultured twice prior to inoculation. Starter culture inoculum was prepared by harvesting 10 mL overnight culture at 4000×g for 15 min, washing with sterilized tap water, and re-suspension of the culture in the original volume. Uniform colony morphology of the overnight inoculum confirmed the purity of the cultures. For the preparation of 10 mL starter culture cocktails, 5 mL of each *L. sakei* FUA 3549/*P. pentosaceus* FUA3550 and *S. carnosus* were mixed.

For the preparation of starter culture with a cocktail of 5 pathogenic strains of *E. coli*, the *E. coli* cocktail was prepared by combining equal volume of washed overnight culture of each of the five strains to form a 5 mL cocktail. *L. sakei* FUA3549 10 mL overnight inoculum was washed and resuspended to 5 mL autoclaved water, and *L. sakei* FUA3549 and *S. carnosus* cocktail was prepared by combining equal volume of washed overnight culture to the final

volume of 5 mL. *E. coli* cocktail was then combined with 5mL of either single strain *L. sakei* FUA3549 or cocktail *L. sakei* FUA3549 and *S. carnosus* to the 10 mL final volume.

2.4 Sausage fermentation

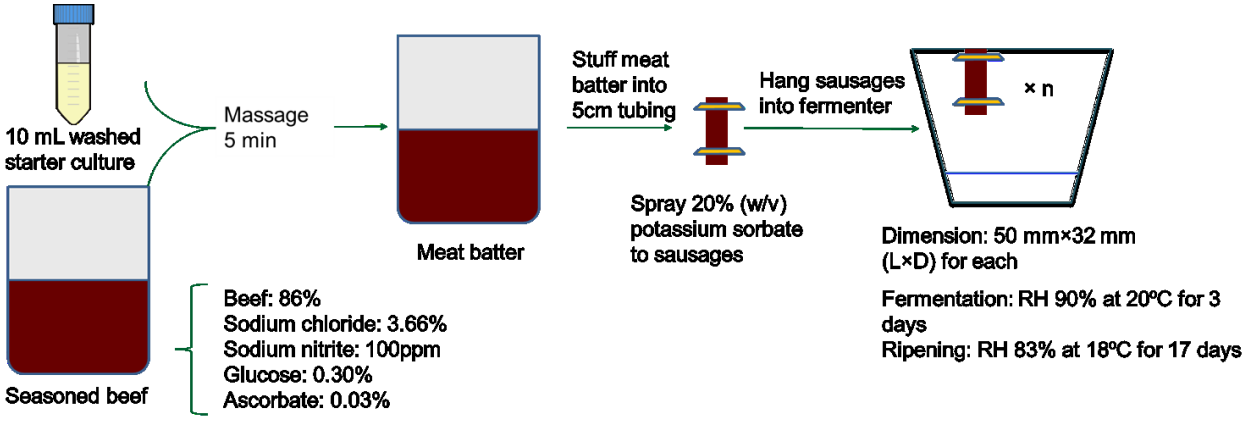

Figure 2. Experimental flow chart of model sausage fermentation.

The sausage batter contained (% wt/wt): 86% ground beef, 3.66% sodium chloride, 0.01% sodium nitrite, 0.3% glucose, 0.03% sodium ascorbate, and 10% inoculum. For the aseptic control, 10 mL solution containing 100 mg L^{-1} each of chloramphenicol, ampicillin, and erythromycin substituted the bacterial inoculum. All handling of meat and sausage batters was carried out in a laminar flow biosafety cabinet; and 70% ethanol was used for product contact surfaces and utensils. Ground beef was prepared from a bottom round of beef after removal of the exterior fat, intermuscular fat, and connective tissues with a sterile knife. The lean beef was cut into steaks, vacuum packaged, and stored at -20°C until use. To prepare the sterile seasoned beef batter, frozen steaks were thawed at 4°C overnight, and then minced with the dry ingredients using a food processer. The seasoned beef batter was portioned and packaged in individual sterile stomacher bags, and stored at -20°C until use. To prepare the beef sausages, the seasoned beef batter was thawed at 4°C overnight, mixed with inoculum or with antibiotics, and massaged in a stomacher. The meat batter was stuffed into dialysis tubing (flat width: 32 mm;

vol/length 3.3 mL/cm, Fisher Scientific, Canada), cut into 5 cm long segments, and closed by plastic closures. Sausage meat batter was maintained at refrigeration temperature during stuffing. Sausages were sprayed with 20% (wt/v) potassium sorbate solution, and sausage weight was recorded. Sausages were hung in a fermenter where the water activity a_W was controlled at 0.90 with saturated barium chloride solution at 20°C or at a_W 0.83 with 2.9 mol kg^{-1} sodium chloride solution at 18°C (Barbosa-Canovas et al., 2007; Franks, 1975). Salt solutions were pre-equilibrated to the designated temperature for 16 h before fermentation. Sausages were incubated at 20°C and relative humidity 90% for 3 d and ripened at 18°C and relative humidity 83% for 17 d. Sausages were sampled on day 0, 1, 3, 4, 11, and 20. Sausage fermentations were carried out in at least triplicate independent fermentations. The sausage weight, water activity (Aqualab, USA), pH and cell counts were analyzed immediately after sample collection. Cell counts of uninoculated seasoned meat was determined by serial dilution and plating on LB agar. Lactobacilli were enumerated by surface plating on MRS agar with pH adjusted to 5.5; staphylococci were enumerated by plating on Mannitol Salt Agar. Total viable and sublethally injured *E. coli* were enumerated by plating on LB agar and Violet Red Bile Agar (VRBA), and incubated at 45°C. Observation of a uniform colony morphology in cell counts of inoculated samples confirmed the identity of fermentation microbiota with the inoculum. Sausage samples were freeze dried, powdered using mortar and pestle, and stored at -20°C.

2.5 Total α-amino nitrogen in fermented sausages

Total α-amino nitrogen was quantified in freeze dried fermented sausages sampled on day 0, 3, and 20 with a modified ninhydrin method (Lie, 1973). In brief, perchloric acid (300 µL 7%) was added to 50 mg freeze dried sample and vortex thoroughly, and the samples were stored at 4°C for 16 h. Samples were centrifuged at 15,000×g for 10 min, and 20 µL potassium chloride

were added to 100 μL supernatant. The supernatant was incubated at 21°C for 1 h, and solids were removed by centrifugation at 15,000×g for 10 min. Reagent 1 (100 μL) and 190 μL sterilized distilled water were added to 10 μL supernatant, and incubated at 100°C using water bath for 16 min. Samples were cooled at room temperature for 20 min, and 500 μL reagent 2 were added into the samples and mixed thoroughly, and the absorbance was read at 570 nm.

2.6 Quantitation of free amino acids in fermented sausages

On day 0, 3, and 20, free amino acids were extracted from freeze dried fermented sausages as described with modifications (Aristoy and Toldra, 1991). Samples (50 mg) were extracted by shaking with 5 mL 0.1M hydrochloric acid at 200 rpm at 4°C for 8 min. The supernatant was collected by centrifugation at 4°C for 20 min, and filtered through a 0.45μm filter (Thermo Scientific, 17mm teflon syringe filter). Perchloric acid (5%, 2 mL) was added to 2 mL and centrifuged at 10,000×g at 4°C for 15 min. The supernatant was analysed by HPLC using β-aminobutyric acid as an internal standard and derivatization with *o*-phthalaldehyde as described (Sedgwick et al., 1991). Amino acid concentrations in sausages were analyzed in three independent fermentation batches for starter culture inoculated samples, and nine independent batches for the aseptic control.

2.7 Fatty acids extraction and quantification

Fatty acids were extracted from freeze dried aseptic control, *L. sakei*, and *L. sakei* and *S. carnosus* sausages sampled on day 0, 3, and 20. Samples (0.1 g) were extracted with 5 mL isopropanol by vortexing for 30s. Supernatant was obtained by centrifugation at 5,000 rpm for 5 min. The remaining fatty acids in the meat pellets were extracted 3 times with 3mL isopropanol and additional 3 times with 3mL hexane/isopropanol (3:2, v/v) mixture. All the supernatants were combined and dried under nitrogen. Extracted samples were analyzed by reversed-phase

liquid chromatography coupled with negative-ion electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS) with ricinoleic acid as the internal standard. Absolute quantification of fatty acids was based on the use of external standards; relative quantification of 2-hydroxyl fatty acids were based on dilution series of 2-OH oleic acid.

2.8 Statistical analysis

Total α-amino nitrogen concentrations, free amino acid concentrations, and fatty acid concentrations were analyzed using nested ANOVA of R (R version 3.5.0) , with strain, time and interactions of strain and time as fixed effect; and fermentation batches as random effect. The principal component analysis (PCA) was carried out by using rotated component correlation matrix model (PASW Statistics 18.0). Results were expressed as mean ± standard error of the means. Significant differences were reported with 5% probability of error ($P < 0.05$).

3. Results

3.1 Establishment of bench-top sausage fermentation protocol

Accumulation of taste-active compounds during sausage ripening influences the taste of the final product; however, sausage fermentation at the pilot scale does not enable the comparison to aseptic controls. The contribution of defined starter cultures and endogenous meat enzymes to accumulation of amino during sausage ripening remains thus uncertain. Aseptic sausages prepared in this study had a cell counts below the detection limit (1 log CFU g^{-1}) throughout fermentation and ripening. Spraying of the exterior of sausage with 20% potassium sorbate was necessary to prevent growth of molds during ripening. The development of bench-top sausage model system is a relatively simple model system for meat fermentations with controlled microbiota, which can be manipulated and applied to various objectives.

3.2 Characterization of fermented sausages

In order to validate the reproducibility of the model system, cell counts, pH, water activity, and moisture loss of sausages were monitored during ripening. The cell counts of uninoculated seasoned beef were below the detection limit. The cell counts of *Lactobacillus* spp. and *Pediococcus* spp. were comparable throughout fermentation.

Cell counts of *S. carnosus* cell counts remained stable around 7.3 log CFU g^{-1} (Fig. 4A). The pH values for both single strain and mixed culture batches dropped from 5.5 to 4.7 during the fermentation stage and increased slightly to 4.9 during further ripening (Fig. 4B). The cell counts for the aseptic control remained below the detection limit of 1 log CFU g^{-1}, and the pH remained stable at 5.5 during 20 days. The moisture loss of dry fermented sausages was about 37% and the corresponding water activity was around 0.87.

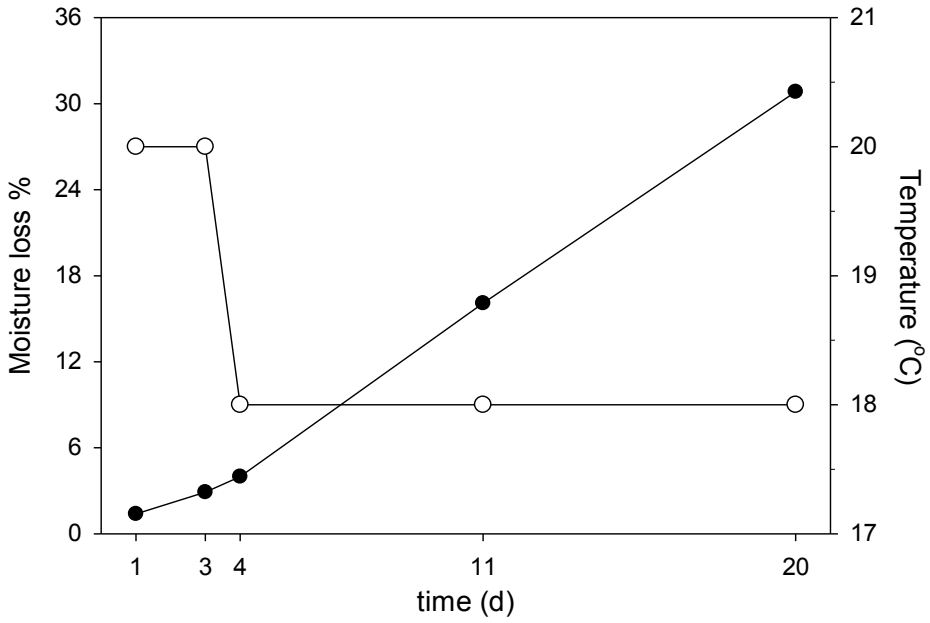

Figure 3. Fermentation temperature and moisture loss in aseptic control sausages. Moisture loss data present means of nine independent fermentations. Open circles (○) indicate temperature condition used for the fermentation, and closed circles (●) indicate moisture loss. (n=9)

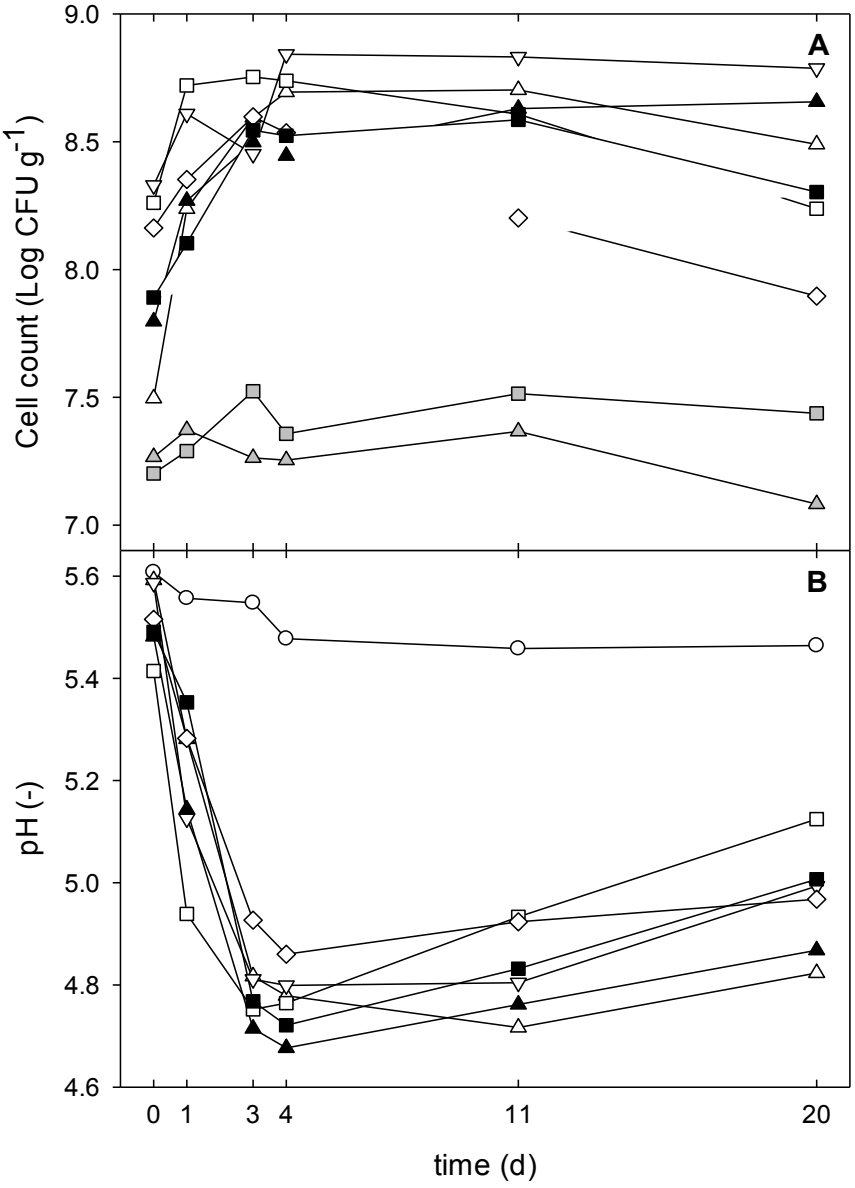

Figure 4. Cell counts (Panel A) and pH (Panel B) of aseptic and inoculated sausages. Cell counts of aseptic controls were below the detection limit of 1 log CFU g^{-1}. Data are presented as means of triplicate biological repeats for inoculated sausages and nine independent fermentations for the aseptic control. **Panel A**, symbols indicate the cell count for sausages fermented with single strain of *L. sakei* FUA3009 (△), *L. plantarum* (▽), *P. pentosaceus* FUA3071 (□), *P. acidilactici* (◇); *L. sakei* FUA3049 and *S. carnosus* (*L. sakei,* ▲; *S. carnosus,* ▲), or *P. pentosaceus* FUA3550 and *S. carnosus* (*P. pentosaceus,* ■; *S. carnosus* ■). **Panel B** shows the dry fermented sausages pH, symbols for the single strain are the same as described. The aseptic control is labelled as (○); *L. sakei* and *S. carnosus* cocktail is labelled as (▲); *P. pentosaceus* and *S. carnosus* cocktail is labelled as (■) (n=3).

3.3 Total α-amino nitrogen concentrations of sausages

Total α-amino nitrogen in aseptic controls and fermented sausages were quantified to assess the proteolytic activity during the sausage fermentation and ripening (Fig. 5). Total α-amino nitrogen concentration for the aseptic control, *P. acidilactici*, and *P. pentosaceus* fermented sausages did not change during 20 days, while the amino nitrogen concentration increased in *L. sakei*, *L. plantarum*, and cocktail inoculated sausages on day 20.

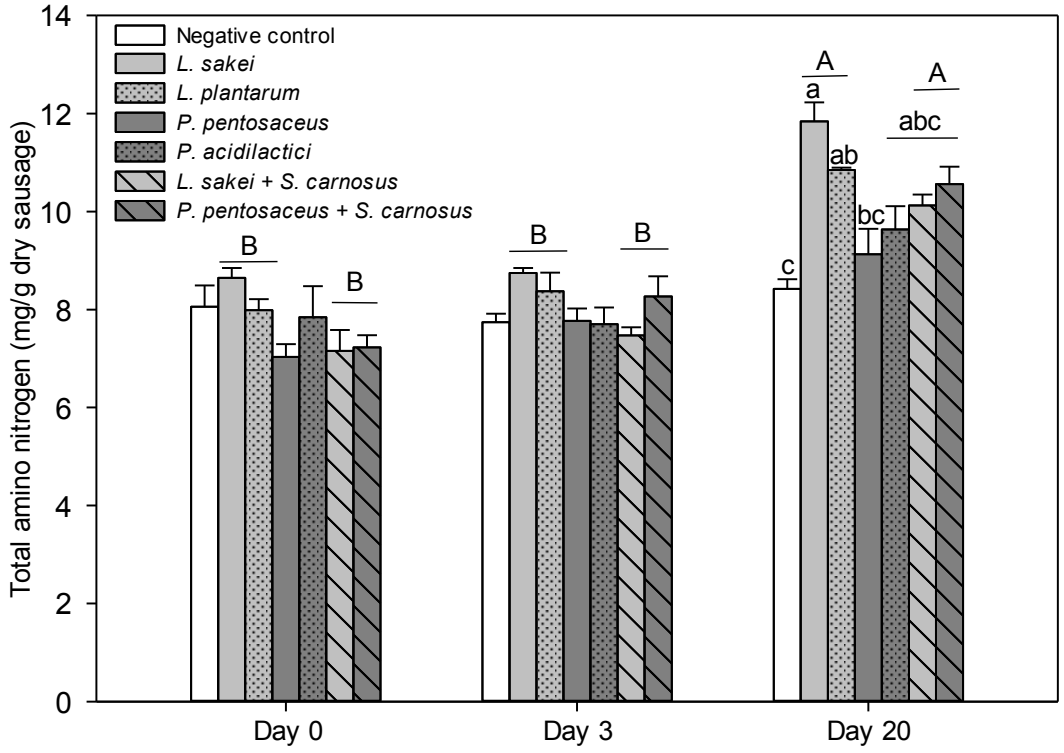

Figure 5. Concentration of total amino nitrogen in dry fermented sausages. Data are presented as means ± standard error of the mean of triplicate biological repeats for the inoculated sausages and nine biological repeats for the aseptic control sausages. Letters A and B denote significant differences (P<0.05) of sausages fermented with the same strains at different time points. Letters a, b, and c denote significant differences (P<0.05) of sausages fermented with different strains at the same time point. Bar colours and patterns indicate aseptic control (white); *L. sakei* (light grey); *L. plantarum* (dotted light grey); *P. pentosaceus* (dark grey); *P. acidilactici* (dotted dark grey); *L. sakei* and *S. carnosus* cocktail (hatched light grey); and *P. pentosaceus* and *S. carnosus* cocktail (hatched dark grey).

Lactobacillus spp. increased the content of total amino nitrogen when compared to the aseptic control (Fig. 5). Inoculation with *S. carnosus* did not accelerate the accumulation of total amino nitrogen during sausage ripening. Overall, the starter cultures exhibited a strain-dependent contribution to proteolytic activity during sausage ripening.

3.4 Free amino acid concentrations of sausages

This study also investigated the contribution of controlled fermentation microbiota to the accumulation of free amino acids during sausage fermentation. Relationships between the free amino acids in sausages fermented with different starter cultures and fermentation time were initially assessed by principle component analysis (Fig. 6).

Fermentation time strongly influenced the accumulation of free amino acids in inoculated sausages, but not in aseptic controls. In keeping with the effect of the respective cultures on accumulation of amino nitrogen, *Lactobacillus* spp. clustered separately from *Pediococcus* spp. when used as single starter cultures. Glutamate, valine, isoleucine, and leucine increased in all treatment groups over time (Table 2). Glutamine concentrations were reduced over time, particularly in *L. sakei* single strain or cocktail fermented sausages during the early stage of fermentation. Glutamate concentrations were higher in all fermented sausages when compared to the aseptic control (Table 2). Fermented sausages had significantly higher concentration of branched chain amino acids (valine, isoleucine, and leucine) when compared to the aseptic control. Arginine concentrations (400-450 mg/100g) were not different in sausages fermented with different strains, or sampled at different fermentation times. The gene coding for glutamate decarboxylase, *gadB,* was present only in *L. plantarum*, but the accumulation of γ-amino butyrate (GABA) by *L. plantarum* was below the detection limit. The concentrations of biogenic

amines histamine, tyramine, cadaverine, putrescine, and 2-phenylethylamine were also below the detection limit in all samples.

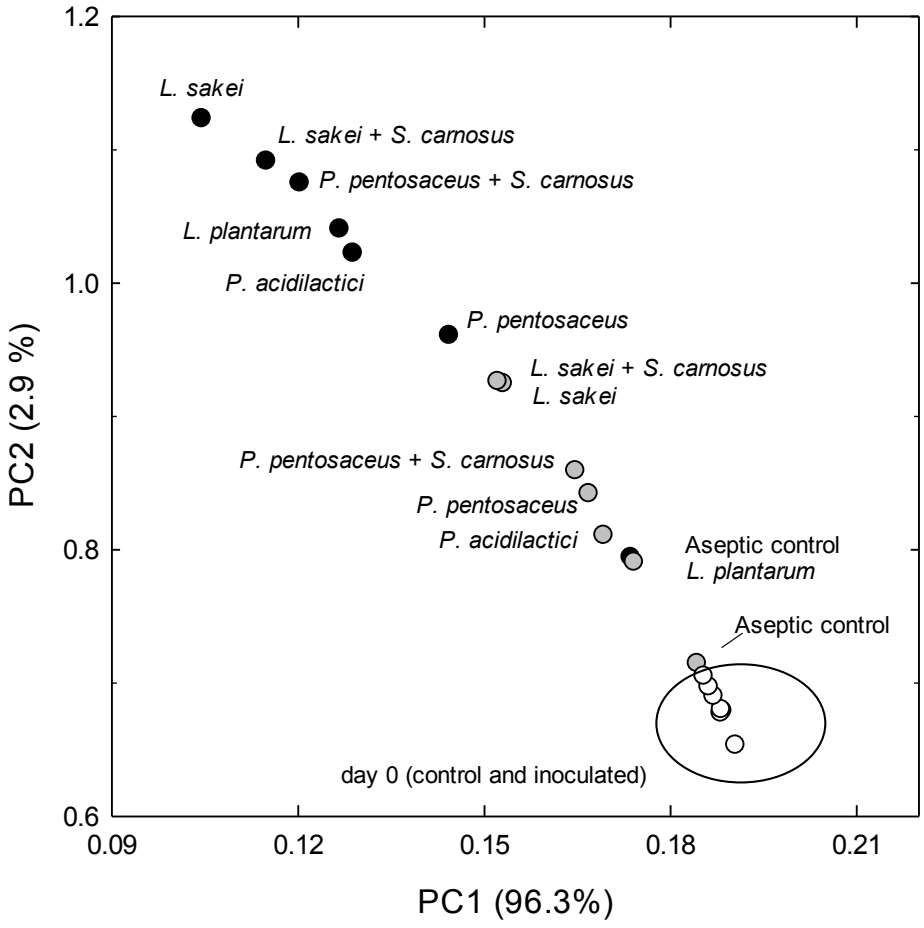

Figure 6. Principal component analysis of free amino acid concentrations in aseptic and inoculated sausages at day 0 (○), day 3 (●), and day 20 (●).

Table 2. Concentration of free amino acids (mg/100g) in dry sausages fermented with different combination of starter cultures

Strain / time (d)	Aseptic control	*L. sakei*	*L. plantarum*	*P. pentosaceus*	*P. acidilactici*	*L. sakei* and *S. carnosus*	*P. pentosaceus* and *S. carnosus*
Glutamate							
0	24±0.40 ^{B}	32±0.80 ^{C}	30±3.1 ^{B}	33±4.6 ^{C}	30±8.1 ^{C}	26±1.0 ^{C}	27±2.7 ^{C}
3	31±1.7 ^{Z,B}	100±5.8 ^{XY,B}	56±6.9 ^{YZ,B}	92±0.98 ^{XY,B}	72±24 ^{XYZ,B}	120±13 ^{X,B}	84±6.4 ^{XYZ,B}
20	57±3.3 ^{Y,A}	170±10 ^{X,A}	140±12 ^{X,A}	140±9.2 ^{X,A}	150±24 ^{X,A}	180±9.2 ^{X,A}	150±1.7 ^{X,A}
Serine							
0	13±1.8 ^{B}	14±1.8	17±1.5	19±0.93	13±3.2	17±1.2 ^{B}	12±1.0
3	16±0.98 ^{B}	6.5±1.5	6.0±1.84	12±0.46	17±4.3	19±2.3 ^{B}	8.5±1.8
20	27±2.5 ^{XY,A}	19±1.5 ^{XY}	10±1.1 ^{Y}	25±4.1 ^{XY}	20±11 ^{XY}	39±4.1 ^{X,A}	24±3.8 ^{XY}
Glutamine							
0	38±2.9	51±3.0 ^{A}	59±3.6	41±10	39±14	35±4.1 ^{A}	27±3.3
3	37±3.9 ^{XY}	29±2.8 ^{XY,B}	48±1.6 ^{X}	26±8.3 ^{XY}	26±11 ^{XY}	11±2.8 ^{Y,B}	20±2.2 ^{XY}
20	29±2.8	25±1.7 ^{B}	42±2.6	22±8.9	23±5.2	11±1.1 ^{B}	12±0.55
Alanine							
0	60±4.9	61±3.3 ^{B}	57±3.4 ^{B}	66±5.5	61±7.5	72±1.8	62±2.5
3	70±11	90±5.6 ^{B}	77±2.6 ^{B}	79±15	69±12	88±6.8	87±8.4
20	85±8.7 ^{Y}	190±11 ^{X,A}	170±21 ^{XY,A}	120±34 ^{XY}	140±62 ^{XY}	150±3.8 ^{XY}	140±1.6 ^{XY}
Tyrosine							
0	22±8.7	12±1.6	12±1.1	47±35	46±35	16±3.6	15±3.5
3	26±6.4	24±3.0	22±0.14	58±38	110±79	32±5.7	29±5.1
20	38±9.2	51±4.1	45±1.7	98±65	120±93	45±1.5	38±4.3
Methionine							
0	18±6.4 ^{AB}	13±3.9 ^{B}	18±4.6 ^{B}	16±2.1	12±1.7 ^{B}	14±1.3 ^{B}	16±1.1 ^{B}
3	18±1.0 ^{B}	22±2.5 ^{AB}	22±2.0 ^{B}	27±1.6	20±3.2 ^{AB}	25±4.2 ^{AB}	26±1.7 ^{AB}
20	35±4.2 ^{A}	49±4.0 ^{A}	53±5.9 ^{A}	42±2.2	41±4.0 ^{A}	51±1.9 ^{A}	47±1.3 ^{A}
Valine							
0	12±0.87 ^{B}	13±1.0 ^{C}	18±2.5 ^{B}	15±2.1 ^{C}	13±2.2 ^{B}	12±2.9 ^{C}	13±0.98 ^{C}
3	17±1.1 ^{Y,B}	30±1.9 ^{XY,B}	24±1.7 ^{XY,B}	31±2.1 ^{XY,B}	25±3.6 ^{XY,B}	28±2.4 ^{XY,B}	32±3.7 ^{X,B}
20	30±1.3 ^{Y,A}	65±5.5 ^{X,A}	59±5.0 ^{X,A}	56±3.8 ^{X,A}	50±4.2 ^{X,A}	56±2.6 ^{X,A}	50±0.66 ^{X,A}
Isoleucine							
0	15±2.2 ^{C}	13±1.4 ^{C}	13±1.7 ^{B}	17±1.1 ^{B}	19±1.9 ^{B}	17±0.99 ^{B}	15±0.73 ^{B}
3	24±1.9 ^{B}	28±0.20 ^{B}	26±3.6 ^{B}	31±2.1 ^{B}	25±3.4 ^{B}	27±1.9 ^{B}	29±1.4 ^{B}
20	40±4.0 ^{Y,A}	63±4.2 ^{X,A}	63±7.7 ^{X,A}	58±4.0 ^{XY,A}	61±3.5 ^{X,A}	56±2.8 ^{X,A}	54±0.94 ^{X,A}
Leucine							
0	28±4.1 ^{C}	26±1.1 ^{C}	29±4.5 ^{C}	35±4.1 ^{C}	38±5.2 ^{B}	33±3.8 ^{C}	32±1.1 ^{C}
3	47±3.2 ^{B}	72±2.0 ^{B}	66±8.8 ^{B}	76±4.0 ^{B}	55±8.9 ^{B}	66±5.4 ^{B}	72±4.5 ^{B}
20	86±9.6 ^{Y,A}	150±4.7 ^{X,A}	150±17 ^{X,A}	140±8.0 ^{X,A}	130±1.8 ^{X,A}	140±4.6 ^{X,A}	130±1.1 ^{X,A}

Data represent means ± standard error of mean of triplicate independent fermentations. Superscripts X, Y, and Z denote significant differences ($P < 0.05$) among concentrations of the same free amino acid in dry sausages fermented with different strains at the same fermentation time; A, B, and C denote significant differences ($P < 0.05$) among concentrations of the same amino acid in dry sausages fermented with the same strain over time. Superscripts are not indicated if values were not significantly different. (n=9 for aseptic control; n=3 for the rest)

3.5 Free fatty acids and hydroxyl fatty acids quantitation in fermented sausages

Changes of fatty acids and hydroxyl fatty acids in dry fermented sausages were quantified to understand the effect of starter culture to the metabolism of fatty acids during sausage fermentation and ripening (Table 3 and Fig. 7). All samples regardless of the starter culture inoculation had an increase of total free fatty acids concentration during ripening (Table 2). Accumulation of saturated fatty acids was time dependent, and the metabolisms of linoleic acid, linolenic acid, and hydroxy fatty acids were starter culture depended (Table 2). *L. sakei* fermented sausages had a significantly higher content of coriolic acid when compared to the aseptic control sausages. For the relative quantitation of 2-OH fatty acids, *L. sakei* and *S. carnosus* had a pronounced increase of overall 2-OH fatty acids when compared to the single strain and the aseptic control (Fig. 7).

Table 3. Concentration of free fatty acids in sausages fermented with different starter cultures

Free Fatty acids (mg/100 g DM)	Time (d)	Aseptic control	*L. sakei*	*L. sakei* and *S. carnosus*
Saturated fatty acids	**0**	90±14 ^{B}	120±40 ^{B}	69±23 ^{C}
	3	130±7.0 ^{B}	90±43 ^{B}	110±4.4 ^{B}
	20	220±0.77 ^{A}	240±7.3 ^{A}	190±15 ^{A}
C14:1 (Myristoleic acid)	**0**	2.1±1.1	2.3±1.2	2.3±1.2
	3	3.4±0.14	2.3±1.2	2.4±1.2
	20	4.8±0.20	4.5±0.31	5.2±0.90
C16:1 (Palmitoleic acid)	**0**	4.0±0.67 ^{B}	5.6±0.49 ^{B}	3.6±0.11 ^{B}
	3	7.1±0.25 ^{B}	4.2±2.0 ^{B}	6.5±0.71 ^{B}
	20	17±0.79 ^{A}	16±1.1 ^{A}	11±1.8 ^{A}
C18:1 (Oleic acid)	**0**	31±6.2 ^{B}	46±5.6 ^{B}	25±1.5 ^{B}
	3	63±4.8 ^{B}	37±19 ^{B}	53±7.4 ^{B}
	20	160±14 ^{A}	150±8.3 ^{A}	104±16 ^{A}
C18:2 (Linoleic acid)	**0**	9.0±2.3 ^{B}	11±0.56 ^{B}	7.1±0.33
	3	14±1.1 ^{B}	8.4±3.9 ^{B}	12±3.1
	20	39±4.7 ^{A}	34±3.0 ^{A}	19±5.5
C18:3 (Linolenic acid)	**0**	0.57±0.29 ^{B}	0.94±0.17	0.90±0.10
	3	1.3±0.14 ^{B}	0.78±0.36	1.3±0.11
	20	3.0±0.29 ^{A}	1.9±0.36	1.2±0.38
mono-OH C18:0	**0**	0±0	0±0	0±0
	3	0±0	0±0	0±0
	20	0±0	0.60±0.12	1.5±0.43
2-OH C18:1 (2-OH oleic acid)	**0**	0±0	0±0	0±0
	3	0±0	0±0	0±0
	20	0±0	0±0	12±3.4
13-OH C18:2 (coriolic acid)	**0**	0.41±0.054	0.67±0.11 ^{B}	0.28±0.025
	3	0.58±0.12	0.29±0.24 ^{B}	0.34±0.053
	20	0.99±0.17 ^{Y}	1.9±0.22 ^{A,X}	0.57±0.047 ^{Y}
Total FA	**0**	140±25 ^{B}	190±13 ^{B}	110±2.3 ^{B}
	3	220±72 ^{B}	140±68 ^{B}	190±18 ^{B}
	20	450±44 ^{A}	440±16 ^{A}	440±43 ^{A}

Data represent means ± standard error of mean of triplicate independent fermentations. Superscripts A, B, C denote significant differences ($P < 0.05$) among concentrations of the same fatty acid in dry sausages with same strain over time. Superscripts X, Y, Z denote significant differences ($P<0.05$) among concentrations of the same fatty acid with different starter cultures at the same time. Superscripts are not indicated if values were not significantly different.

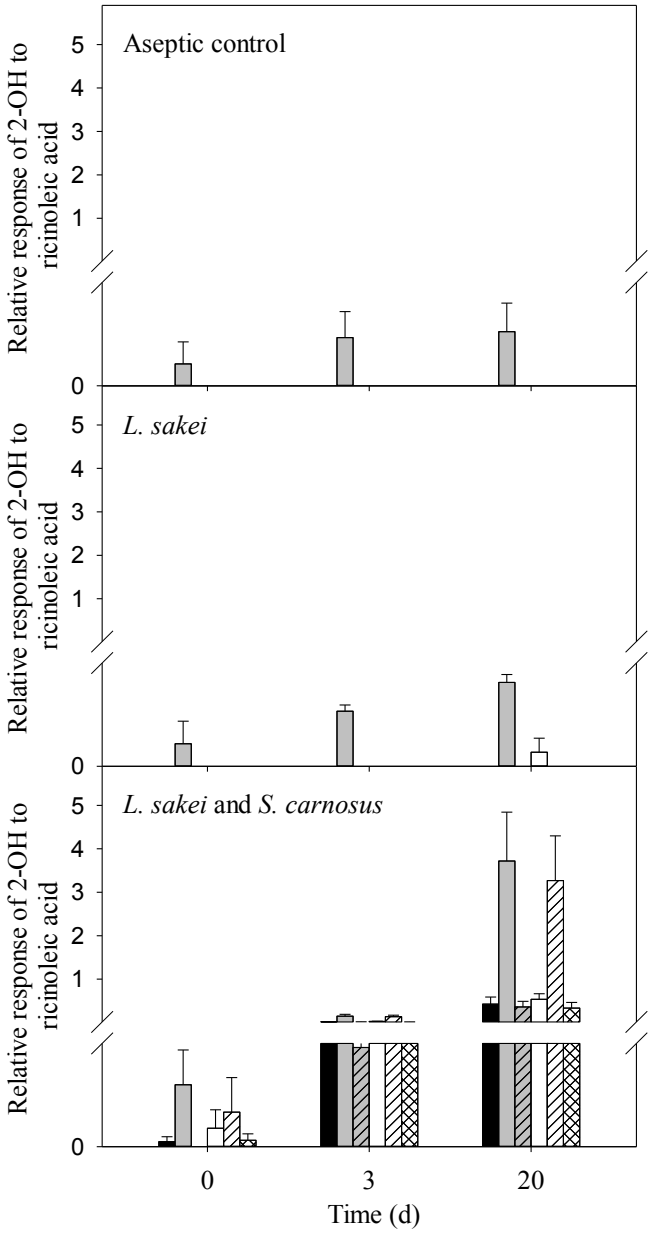

Figure 7. Relative response of 2-hydroxyl fatty acids in fermented sausages to the internal standard ricinoleic acid. Bar colours indicate relative quantitation of 2-OH C14:0 (black), 2-OH C16:0 (grey), 2-OH C16:1 (grey hatched), 2-OH C18:0 (white), 2-OH C18:1 (white hatched), 2-OH C18:2 (white and crossed).

3.6 Survival of pathogenic *E. coli* in fermented sausages

The effect of starter culture and processing condition on the survival of *E. coli* was investigated with a cocktail of 5 pathogenic strains. *L. sakei* FUA3549 was used as starter culture, alone or in combination with *S. carnosus*. The addition of the *E. coli* strain cocktail did not influence cell counts of the starter cultures or the pH of the sausages (Fig. S2). Cell counts of *E. coli* were monitored on LB and VRB agars to identify total *E. coli* including sublethally injured cells. The initial cell counts for *E. coli* cocktail in all treatments were around 6.8 log CFU g^{-1}. Cell counts were reduced by less than 0.5 log CFU g^{-1} on day 20 (Fig. 8). Cell counts obtained on LB and VRB agars differed by 0.5-1 log CFU g^{-1} after 20 d of fermentation, indicating sublethal injury of surviving cells. The presence of starter cultures did not affect survival of *E. coli*; the moderate reduction of *E. coli* observed cell counts on day 20 was observed in fermented samples as well as the aseptic control.

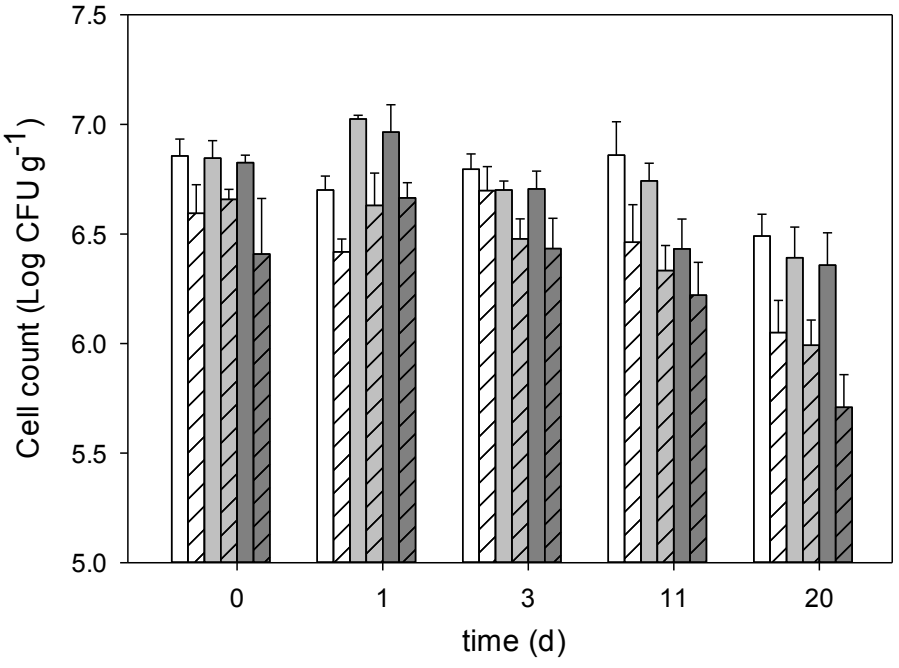

Figure 8. Cell counts of pathogenic *E. coli* in aseptic controls and sausages fermented with *L. sakei* and *S. carnosus*. Data are presented as means ± standard error of the mean of triplicate

independent experiments. Bar colours indicate *E. coli* cell counts in aseptic controls (white), sausages fermented with *L. sakei* FUA3549 (light grey), or sausages fermented with *L. sakei* FUA3549 and *S. carnosus* (dark grey). Plain bars represent *E. coli* cell counts on LB plates; hatched bars represent *E. coli* cell count on VRBA plates. Cell count and pH for the single strain *L. sakei* or the cocktail *L. sakei* and *S. carnosus* were shown in Figure S2.

4. Discussion

4.1 Development of sausage model system

Sausage model systems have previously been used to study the effect of starter culture on proteolysis (López et al., 2015a; López et al., 2015b; Molly et al., 1997). The use of aseptic meat in a sausage model system in this study enabled unprecedented control of microbiota throughout the fermentation and ripening period; aseptic controls had no detectable microbiota throughout the 20 d ripening period. Surface molds were controlled by dipping in 20% potassium sorbate (Holley, 1981). In industrial practice, smoking is commonly used to prevent mold development unless products are inoculated with a mold culture for surface ripening (Holley, 1981). The ripening conditions used in the model system matched slow fermentation at low temperature. Ripening at controlled temperature and relative humidity provided a consistent product quality; despite the difference in caliber of the mini-sausages when compared to commercial products, physiochemical properties were comparable to studies reporting water activity and pH of dry cured sausages (Hagen et al., 1996; Ordóñez et al., 1999).

4.2 The effect of endogenous proteolysis and accumulation of free amino acids

The generation of amino acids in fermented sausage is attributed to the muscle proteinases and microbial proteases. Meat proteases cathepsin (B, D, H, and L) degrade myosin and actin to peptides, microbial peptidases further degrade peptides to amino acids (Ordóñez et al., 1999; Toldrá et al., 1992; Toldrá and Flores, 1998). Cathepsin B, L, D are mainly active during fermentation stage, and their activity is reduced by the low water activity during ripening

(Toldrá et al., 1992). The present study observed that the accumulation of total amino nitrogen did not differ between inoculated sausages and aseptic controls after 3 d, however, the accumulation of specific amino acids at 3 d was attributable to bacterial aminopeptidases (Fig. 5, 4, and Table 2).

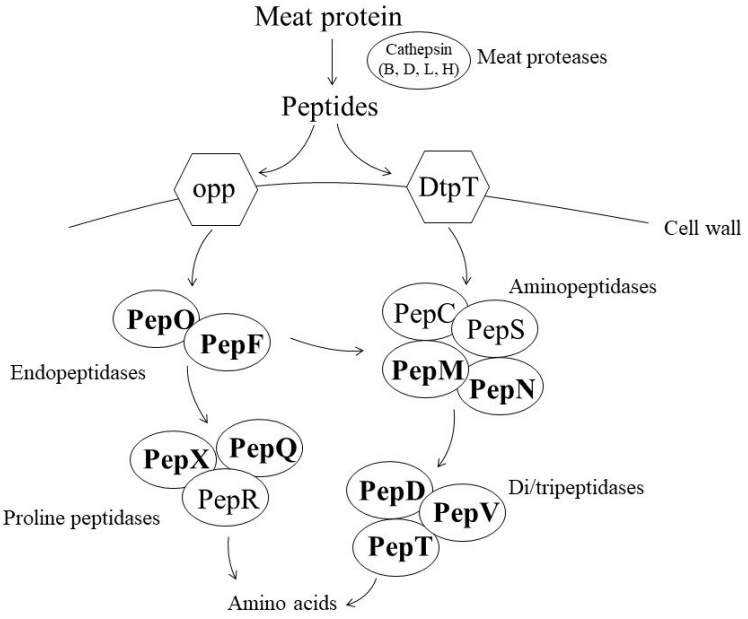

Figure 9. Schematic overview of the peptides utilization by L. sakei and P. acidilactici during sausage fermentation. The drawing is based on information on peptidases in *L. sakei* 23K and *P. acidilactici* DSM 20284^T provided by Sinz and Schwab (2012), Toldrá (1998), and Zheng et al. (2015). All the peptidases were identified in *L. sakei* 23K and only bolded peptidases were identified in *P. acidilactici* DSM 20284^T.

Further decomposition of peptides to free amino acids by bacterial peptidases occurred during the ripening stage, resulting in higher accumulation of total α-amino nitrogen in inoculated sausages when compared to the aseptic controls (Freiding et al., 2011; Molly et al., 1997). Peptidases PepC, PepN, and PepM and proline peptidases PepX and PepQ are encoded in genomes of most lactobacilli but the activity of other peptidases is strain or species specific (Liu et al., 2010). Dipeptides are preferred over amino acids and tetrapeptides by the cellular transport system and peptidases (Sinz and Schwab, 2012). Amino acid analysis revealed that the

accumulation of free amino acids in sausages with controlled microbiota strongly depended on the type of starter and the ripening time (Fig, 3, Fig. 6, Fig. 9, and Table 2).

The use of the model system provided evidence that bacterial enzymes influenced the release of free amino acids, even during the initial fermentation stage. Among amino acids, glutamate is particularly relevant because it imparts umami, savoury flavour with a taste threshold of approximately 1 mmol kg^{-1} (Toelstede and Hofmann, 2008; Zhao et al., 2015). In cheese and cereal fermentations, the conversion of glutamine to glutamate or GABA depends strain specific enzyme activities of lactobacilli; glutamate accumulation by starter cultures strongly imparts the taste of bread and cheese (Toelstede and Hofmann, 2008; Zhao et al., 2015). Glutamate also impacts flavour or fermented meats (Jurado et al., 2007); however, factors influencing accumulation of glutamate in sausage fermentation remain unclear. The present study demonstrates bacterial activity accumulates glutamate to concentrations that are more than 10 fold in excess of the taste threshold. Different from sourdough fermentation, differences in glutamate accumulation between strains were minor and not attributable to glutamate decarboxylase activity (Zhao et al., 2015). Decarboxylation of glutamate by glutamate decarboxylase improves acid resistance of lactobacilli (Su et al., 2011). The pH optimum of GAD from *Lactobacillus brevis* is pH 4.2 (Cotter et al., 2003); moreover, GAD expression in *L. reuteri* required low pH (Teixeira et al., 2014). The lowest pH during dry sausages fermentation, pH 4.7, may have been too high to trigger GAD expression by *L. plantarum*.

Several species of lactobacilli utilize arginine as an alternative energy source via arginine-deiminase (ADI) pathway, which contributes to pH homeostasis and acid tolerance of lactobacilli (Gänzle et al., 2007). The current study did not observe a pronounced arginine uptake either in aseptic or inoculated sausages. *L. sakei* is ADI positive, but arginine utilization did not

confer a competitive advantage to *L. sakei* during sausage fermentation (Zheng et al., 2015; Zúñiga et al., 2002).

The branched-chain amino acids leucine, isoleucine, and valine are degraded to branched aldehydes, alcohols and acids by *S. xylosus* and *S. carnosus* (Stahnke et al., 2002). Addition of *S. carnosus* to dried sausages accelerated sausage maturation, and the maturity correlated significantly with metabolism of branched chain amino acids metabolism (Stahnke et al., 2002). However, addition of *S. carnosus* to sausages fermented with *L. sakei* FUA3549 and *P. pentosaceus* FUA3550 did not accelerate the utilization of valine, isoleucine, and leucine, which may relate to the short ripening time, or to strain specific differences (Olesen et al., 2004).

4.3 Biogenic amines in fermented sausages

Biogenic amines in fermented meats are products of microbial decarboxylation of amino acids. Histamine, putrescine, cadaverine, tyramine, 2-phenylethylamine, spermine, and spermidine are most prominent biogenic amines in fermented sausages (Shalaby, 1996; Silla Santos, 1996). Their concentration in fermented sausages depends on the hygienic quality of the raw material and the activity of fermentation microbiota (Suzzi and Gardini, 2003). Enterococci and *Enterobacteriaceae* accumulated tyramine, cadaverine and putrescine in fermented sausages (Bover-Cid, 2000a; Roig-Sagués et al., 1999). The use of selected decarboxylase negative starter cultures, *L. sakei*, *S. carnosus* and *S. xylosus*, reduced concentrations of putrescine, cadaverine, and tyramine in sausages by about 90% (Bover-Cid, 2000b). The use of clean meat and decarboxylase negative starter cultures in the present study prevented accumulation of biogenic amines, in keeping with prior knowledge on the role of meat quality and fermentation microbiota on formation of biogenic amines in fermented meats.

4.4 Effect of starter culture to the accumulation of fatty acids

Fat is a major ingredient (10%-40%) in dry fermented sausages, and free fatty acids generated from lipolysis during sausages ripening play an important role in developing the typical cured aroma (Vignolo et al., 2010). Endogenous muscle and adipose tissue lipases dominates the release of free fatty acids from triglycerides during sausage fermentation (Toldrá, 1998). Free fatty acids are further converted to esters, aldehydes, ketones, lactones, and alcohols through chemical oxidation and bacterial metabolism (Flores and Toldrá, 2011; Zuber and Horvat, 2007). The increase of total free fatty acids in dry sausages was time dependant but not significantly different among samples, confirming the predominant role of endogenous lipase in free fatty acid release (Table 3; Casaburi et al., 2007; Casaburi et al., 2008; Zuber and Horvat, 2007). Unsaturated fatty acids are chemically oxidized to lipid peroxides with the presence of oxygen, and further degradation of lipids to aldehydes and ketones results in the development of oxidative rancidity (Ladikos and Lougovois, 1990).

Under aerobic conditions, some lactic acid bacteria including *L.* sakei, *L. plantarum*, *P. acidilactici* produce hydrogen peroxide (Borch and Molin, 1989; Whittenbury, 1964). Therefore, catalase positive *S. carnosus* and *S. xylosus* are commonly used as addition to lactobacilli for meat starter culture to prevent off-flavour (Hammes and Hertel, 1998). *L. sakei* and *L. plantarum* also have catalases, and their ability to form catalase is independent from formation of hydrogen peroxide (Hertel et al., 1998; Whittenbury, 1964). Catalase positive *S. carnosus* has the antioxidant properties which reduce the oxidation of linoleic acid to coriolic acid (Table 3). Additional to the catalase activity, *S. carnosus* lipase activity is essential for the development of flavour during ripening (Hammes and Knauf, 1994). Microbial lipase has a relatively minor contribution to the overall lipolysis phenomena during sausage ripening (Hammes and Knauf,

1994), the addition of *S. carnosus* did not accelerate the release of fatty acids during fermentation (Table 3).

Linoleate hydratases in *L. plantarum* convert linoleic acid to antifungal 10-hydroxy-12-octadecenoic acid and 13-hydroxy-9-octadecenoic acid, and the production of 10-hydroxy-12-octadecenoic acid altered the cell membrane hydrophobicity (Chen et al., 2016). Hydratase positive *Lactobacillus hammesii* and *Lactobacillus sanfranciscensis* produced coriolic acid (13-OH C18:2) which demonstrated antifungal activity at 0.15% (wt/wt) in sourdough bread (Black et al., 2013). The genome of *L. sakei* 23k harbours a linoleate hydratase which is 79% identical to the linoleate hydratase in *L. plantarum* and likely contributes to formation of hydroxy fatty acids. Multiple mono-OH C18:2 fatty acids were identified in *L. sakei* dry sausages (data not shown), thus the accumulation of coriolic acid in fermented and aseptic sausage may also result from chemical oxidation of linoleic acid, or from oxidation by meat lipoxygenase (Table 2; Kim and Oh, 2013). *L. sakei* dry sausage had the highest concentration of coriolic acid, but the amount was lower than the minimum inhibitory concentration (0.3 g L^{-1}) required for antifungal activity (Liang et al., 2017).

Microbial cytochrome P450 enzyme α-hydroxylases catalyse both saturated and unsaturated fatty acids (C12-C18) carbonyl terminal α- or β-positions to produce 2-hydroxy or 3-hydroxyl fatty acids (Kim and Oh, 2013). *Sphingomonas paucimobilis* has α-hydroxylases (Matsunaga et al., 2000), but *Staphylococcus* spp. P450 is 39% identical and a quarter length to *S. paucimobilis* α-hydroxylase, and not present in *S. carnosus* based on NCBI protein blast. Yeast P450 enzyme ω-hydroxylase catalyze terminal or subterminal fatty acid (ω-2 position) hydroxylation (Kim and Oh, 2013). Yeast and *S. carnosus* break down fatty acids by β-oxidation, which is a cyclic oxidation system and yield high energy for cell growth, and the 3-

OH fatty acid can be formed by incomplete β-oxidation (Endrizzi et al., 1996; Leroy et al., 2006).

4.5 Survival of pathogenic *E. coli* during dry sausage fermentation

Pathogenic *E. coli* survive during the processing of dry fermented sausages (Holck et al., 2011; Muthukumarasamy and Holley, 2007; Nissen and Holck, 1998; Rode et al., 2012b). *E. coli* O157:H7 has long been considered as prototype for EHEC and most studies on EHEC survival in sausages used strains of this serotype (Holck et al., 2011). Other serotypes of EHEC were considered only after six additional serotypes, O26, O103, O45, O111, O121, and O145, were recognized as significant contributors to foodborne EHEC infections in the U.S. (Balamurugan et al., 2017). The pathogenic strain cocktail used in the present study was composed on the basis of the resistance of more than 100 strains of Shiga-toxin producing *E. coli* to heat and pressure (Garcia-Hernandez et al., 2015; Liu et al., 2015). The use of either single strain or mixed starter cultures did not have a significant impact on the cell reduction of *E. coli* strain cocktail when compared to aseptic controls. Cell counts of *E. coli* in sausages were reduced by less than 1 log CFU g^{-1}, a cell count reduction within the range of previous reports on the fate of *E. coli* O157 (Holck et al., 2011). Enumerating *E. coli* on selective media only, however, does not account for sublethal injured cells and underestimates the viable cell counts (Muthukumarasamy and Holley, 2007; Nissen and Holck, 1998). The aseptic sausage model system enabled the use of both non-selective and selective media to demonstrate that total viable cells of *E. coli* were reduced by 0.5 log CFU g^{-1} only. This exceptional recovery of pathogenic *E. coli* may relate to the unprecedented use of a strain cocktail in combination with enumeration on non-selective media (Holck et al., 2011). A reduction of pathogenic *E. coli* by more than 5 log CFU g^{-1} is required to meet the regulatory requirements in some countries including Canada and the U.S. (Holck et al.,

2011). Post-process heating is an effective approach to achieve a 5 log reduction of cell counts in dry fermented sausages (Rode et al., 2012b). Alternatively a long time ripening of 5.5 month at 4^{o}C completely eliminated *E. coli* O157:H7 in dry fermented sausages (Nissen and Holck, 1998). *L. monocytogenes* survive better under cold environment; the use of bacteriocin producing starter cultures has a more profound effect on reduction of *L. monocytogenes* than a reduction of the pH (Lahti et al., 2001). The aseptic sausage model developed in the present study provides an excellent tool for further studies on the role of starter cultures and process conditions on the survival of pathogenic bacteria during production of dry cured sausages.

5. Conclusion

In conclusion, this study developed a sausage model system, which can be manipulated and used for varies objectives. The uses of aseptic meat and defined starter cultures enabled control of fermentation microbiota throughout 20 d of fermentation and ripening. The accumulation of free amino acids was strongly correlated with ripening time; however, glutamate accumulation was not dependent on strain-specific properties (Fig. 6; Table 2). We did not observe a significant impact of starter cultures to the metabolism of free amino acids. Accumulation of free fatty acids at the end of ripening was a result of endogenous lipolysis, and unsaturated fatty acids were further utilized by starter cultures. Consistent with the literature, sausage fermentation and ripening for less than 20 d did not reduce cell counts of pathogenic *E. coli* by more than 1 log CFU g^{-1}, necessitating further studies on the control of *E. coli* in fermented meats.

6. Future directions

The current study developed a sausage model system, and the fermentation microbiota was controlled by using sterile meat and potassium sorbate spray. Cell counts for the aseptic

control sausages were below the detection limits throughout 20 d fermentation process, and the pH maintained consistent 5.5 (Fig. 4). Since the aseptic control in the current study was not acidified, glucono delta-lactone (GDL), which is a food additive for sausage acidification, is suggested to use to acidify the aseptic control sausage to match the starter culture inoculated sausage acidity (Maijala et al., 1993). Dry cured sausages in the current study were ripened for 17 d, and the metabolism of branched chain amino acids by *S. carnosus* was not pronounced because of the relatively short ripening period. Therefore, future studies should consider to extend the ripening time. The accumulation of taste active amino acids was strain depended (Table 2), so trained panel sensory study on flavour intensity is recommend for further investigation. The γ-glutamyl dipeptide exerts kokumi activity, which can enhance the perceived flavour intensity of other tastants (Ueda et al., 1997). Accumulation of γ-glutamyl dipeptide is strain specific, and sourdough fermented with γ-glutamyl dipeptide accumulating *L. reuteri* enhanced the salty taste of bread (Zhao and Gänzle, 2016). Therefore, evaluating the effect of starter culture to γ-glutamyl dipeptide accumulation on sausage sensory properties will provide assistance for meat starter culture selection and development. The current 20 d sausage fermentation did not reduce pathogenic *E. coli* for 1 log (Fig. 8); therefore, investigations on processing strategies to reduce pathogenic *E. coli* are subject future studies.

References

Aristoy, M.C., Toldra, F., 1991. Deproteinization techniques for HPLC amino-acid-analysis in fresh pork muscle and dry-cured ham. J. Agric. Food Chem. 39, 1792-1795.

Balamurugan, S., Ahmed, R., Gao, A., Strange, P., 2017. Comparison of the fate of the top six non-O157 shiga-toxin producing *Escherichia coli* (STEC) and *E. coli* O157:H7 during the manufacture of dry fermented sausages. Int. J. Food Microbiol. 259, 14-21.

Barbosa-Canovas, G., Schmidt, S.J., Fontana, A.J., 2007. Water Activity in Foods: Fundamentals and Applications, Wiley-Blackwell, Hoboken.

Black, B.A., Zannini, E., Curtis, J.M., Gänzle, M., 2013. Antifungal hydroxy fatty acids produced during sourdough fermentation: microbial and enzymatic pathways, and antifungal activity in bread. Appl. Environ. Microbiol. 79, 1866-1873.

Borch, E., Molin, G., 1989. Aerobic growth and product formation of *Lactobacillus*, *Leuconostoc, Brochothrix*, and *Carnobacterium* in batch cultures. Appl. Microbiol. Biotechnol. 30, 81-88.

Bover-Cid, S., Schoppen, S., Izquierdo-Pulido, M., Vidal-Carou, M.C.,1999. Relationship between biogenic amine contents and the size of dry fermented sausages. Meat Sci. 51, 305-311.

Bover-Cid, S., 2000a. Influence of hygienic quality of raw materials on biogenic amine production during ripening and storage of dry fermented sausages. J. Food Prot. 63, 1544-1550.

Bover-Cid, S., 2000b. Mixed starter cultures to control biogenic amine production in dry fermented sausages. J. Food Prot. 63, 1556-1562.

Casaburi, A., Aristoy, M., Cavella, S., Di Monaco, R., Ercolini, D., Toldrá, F., Villani, F., 2007. Biochemical and sensory characteristics of traditional fermented sausages of Vallo di Diano (Southern Italy) as affected by the use of starter cultures. Meat Sci. 76, 295-307.

Casaburi, A., Di Monaco, R., Cavella, S., Toldrá, F., Ercolini, D., Villani, F., 2008. Proteolytic and lipolytic starter cultures and their effect on traditional fermented sausages ripening and sensory traits. Food Microbiol. 25, 335-347.

Chen, Y.Y., Liang, N.Y., Curtis, J.M., Gänzle, M.G., 2016. Characterization of linoleate 10-hydratase of *Lactobacillus plantarum* and novel antifungal metabolites. Front. Microbiol. 7, 1561.

Cordeiro, R.P., Luciano, F.B., Holley, R.A., 2013. Evaluation of deodorized yellow mustard concentrations for control of *Escherichia coli* O157:H7 viability in dry fermented sausage. Food Control. 33, 20-24.

Cotter, P., Hill, C., Cotter, P., 2003. Surviving the acid test: Responses of gram-positive bacteria to low pH. Microbiol. Mol. Biol. Rev. 67, 429-453.

Ducic, M., Klisara, N., Markov, S., Blagojevic, B., Vidakovic, A., Buncic, S., 2016. The fate and pasteurization-based inactivation of *Escherichia coli* O157, *Salmonella* Typhimurium and *Listeria monocytogenes* in dry, fermented sausages. Food Control. 59, 400-406.

Endrizzi, A., Pagot, Y., Le Clainche, A., Nicaud, J.M., Belin, J.M., 1996. Production of lactones and peroxisomal beta-oxidation in yeasts. Crit. Rev. Biotechnol. 16, 301-329.

Fadda, S.G., Oliver, G., Vignolo, G., 2002. Protein degradation by *Lactobacillus plantarum* and *Lactobacillus casei* in a sausage model system. J. Food Sci. 67, 1179-1183.

Fernando, J. (2017). *Consumer corner: Demand for processed meat*. Retrieved Mar 20, 2018, from https://www1.agric.gov.ab.ca/$Department/deptdocs.nsf/all/sis16293

Flores, M., Toldrá, F., 2011. Microbial enzymatic activities for improved fermented meats. Trends Food Sci.Technol. 22, 81-90.

Franks, F., 1975. Water A Comprehensive Treatise: Aqueous Solutions of Amphiphiles and Macromolecules, Springer US, New York and London.

Freiding, S., Gutsche, K.A., Ehrmann, M.A., Vogel, R.F., 2011. Genetic screening of *Lactobacillus sakei* and *Lactobacillus curvatus* strains for their peptidolytic system and amino acid metabolism, and comparison of their volatilomes in a model system. Syst. Appl. Microbiol. 34, 311-320.

Gänzle, M.G., Vermeulen, N., Vogel, R.F., 2007. Carbohydrate, peptide and lipid metabolism of lactic acid bacteria in sourdough. Food Microbiol. 24, 128-138.

Garcia-Hernandez, R., McMullen, L., Gänzle, M., 2015. Development and validation of a surrogate strain cocktail to evaluate bactericidal effects of pressure on verotoxigenic *Escherichia coli*. Int. J. Food Microbiol. 205, 16-22.

Hagen, B.F., Berdague, J.L., Holck, A.L., Naes, H., Blom, H., 1996. Bacterial proteinase reduces maturation time of dry fermented sausages. J. Food Sci. 61, 1024-1029.

Hammes, W.P., Hertel, C., 1998. New developments in meat starter cultures. Meat Sci. 49, 125-138.

Hammes, W.P., Knauf, H.J., 1994. Starters in the processing of meat products. Meat Sci. 36, 155-168.

Heir, E., Holck, A.L., Omer, M.K., Alvseike, O., Måge, I., Høy, M., Rode, T.M., Sidhu, M.S., Axelsson, L., 2013. Effects of post-processing treatments on sensory quality and Shiga toxigenic *Escherichia coli* reductions in dry-fermented sausages. Meat Sci. 94, 47-54.

Hertel, C., Schmidt, G., Fischer, M., Oellers, K., Hammes, W.P., 1998. Oxygen-dependent regulation of the expression of the catalase gene *katA* of *Lactobacillus sakei* LTH677. Appl. Environ. Microbiol. 64, 1359-1365.

Holck, A.L., Axelsson, L., Rode, T.M., Høy, M., Måge, I., Alvseike, O., L'Abée-Lund, T.M., Omer, M.K., Granum, P.E., Heir, E., 2011. Review: Reduction of verotoxigenic *Escherichia coli* in production of fermented sausages. Meat Sci. 89, 286-295.

Holley, R.A., 1981. Prevention of surface mold growth on Italian dry sausage by natamycin and potassium sorbate. Appl. Environ. Microbiol. 41, 422-429.

Jurado, Á, García, C., Timón, M.L., Carrapiso, A.I., 2007. Effect of ripening time and rearing system on amino acid-related flavour compounds of Iberian ham. Meat Sci. 75, 585-594.

Kim, K., Oh, D., 2013. Production of hydroxy fatty acids by microbial fatty acid-hydroxylation enzymes. Biotechnol. Adv. 31, 1473-1485.

Lachowicz, K., Żochowska-Kujawska, J., Sobczak, M., 2012. Fermentation: effects on food properties, CRC Press, Boca Raton.

Ladikos, D., Lougovois, V., 1990. Lipid oxidation in muscle foods: a review. Food Chem. 35, 295-314.

Lahti, E., Johansson, T., Honkanen-Buzalski, T., Hill, P., Nurmi, E., 2001. Survival and detection of *Escherichia coli* O157:H7 and *Listeria monocytogenes* during the manufacture of dry sausage using two different starter cultures. Food Microbiol. 18, 75-85.

Leroy, F., Verluyten, J., De Vuyst, L., 2006. Functional meat starter cultures for improved sausage fermentation. Int. J. Food Microbiol. 106, 270-285.

Liang, N., Cai, P., Wu, D., Pan, Y., Curtis, J.M., Gänzle, M.G., 2017. High-Speed Counter-Current Chromatography (HSCCC) purification of antifungal hydroxy unsaturated fatty acids from plant-seed oil and *Lactobacillus* Cultures. J. Agric. Food Chem. 65, 11229-11236.

Lie, S., 1973. The EBC-Ninhydrin Method for Determination of Free Alpha Amino Nitrogen. J. Inst. Brewing. 79, 37-41.

Liu, M., Bayjanov, J.R., Renckens, B., Nauta, A., Siezen, R.J., 2010. The proteolytic system of lactic acid bacteria revisited: a genomic comparison. BMC genomics. 11, 1-15.

Liu, Y., Gill, A., McMullen Lynn, Gänzle, M.G., 2015. Variation in Heat and Pressure Resistance of Verotoxigenic and Nontoxigenic *Escherichia coli*. J. Food Prot. 78, 111-120.

López, C.M., Sentandreu, M.A., Vignolo, G.M., Fadda, S.G., 2015a. Low molecular weight peptides derived from sarcoplasmic proteins produced by an autochthonous starter culture in a beaker sausage model. EuPA Open Proteom. 7, 54-63.

López, C.M., Sentandreu, M.A., Vignolo, G.M., Fadda, S.G., 2015b. Proteomic and peptidomic insights on myofibrillar protein hydrolysis in a sausage model during fermentation with autochthonous starter cultures. Food Res. Int. 78, 41-49.

Luciano, F.B., Belland, J., Holley, R.A., 2011. Microbial and chemical origins of the bactericidal activity of thermally treated yellow mustard powder toward *Escherichia coli* O157:H7 during dry sausage ripening. Int. J. Food Microbiol. 145, 69-76.

Maijala, R.L., Eerola, S.H., Aho, M.A., Hirn, J.A., 1993. The effect of GDL-induced pH decrease on the formation of biogenic amines in meat. J. Food Prot. 56, 125-129.

Matsunaga, I., Sumimoto, T., Ueda, A., Kusunose, E., Ichihara, K., 2000. Fatty acid-specific, regiospecific, and stereospecific hydroxylation by cytochrome P450 (CYP152B1) from *Sphingomonas paucimobilis*: Substrate structure required for a-hydroxylation. Lipids. 35, 365-371.

McLeod, A., Måge, I., Heir, E., Axelsson, L., Holck, A.L., 2016. Effect of relevant environmental stresses on survival of enterohemorrhagic *Escherichia coli* in dry-fermented sausage. Int. J. Food Microbiol. 229, 15-23.

Molly, K., Demeyer, D., Johansson, G., Raemaekers, M., Ghistelinck, M., Geenen, I., 1997. Importance of meat enzymes in ripening and flavour generation in dry fermented sausages. First results of a European project. Food Chem. 59, 539-545.

Montel, M.C., Masson, F., Talon, R., 1998. Bacterial role in flavour development. Meat Sci. 49, S123.

Muthukumarasamy, P., Holley, R.A., 2007. Survival of *Escherichia coli* O157:H7 in dry fermented sausages containing micro-encapsulated probiotic lactic acid bacteria. Food Microbiol. 24, 82-88.

Nissen, H., Holck, A., 1998. Survival of *Escherichia coli* O157:H7, *Listeria monocytogenes* and *Salmonella kentucky* in Norwegian fermented, dry sausage. Food Microbiol. 15, 273-279.

Olesen, P.T., Meyer, A.S., Stahnke, L.H., 2004. Generation of flavour compounds in fermented sausagesthe influence of curing ingredients, *Staphylococcus* starter culture and ripening time. Meat Sci. 66, 675-687.

Ordóñez, J.A., Hierro, E.M., Bruna, J.M., Hoz, L.d.l., 1999. Changes in the components of dry-fermented sausages during ripening. Crit. Rev. Food Sci. Nutr. 39, 329-367.

Rode, T.M., Holck, A., Axelsson, L., Høy, M., Heir, E., 2012a. Shiga toxigenic *Escherichia coli* show strain dependent reductions under dry-fermented sausage production and post-processing conditions. Int. J. Food Microbiol. 155, 227-233.

Rode, T.M., Holck, A., Axelsson, L., Høy, M., Heir, E., 2012b. Shiga toxigenic *Escherichia coli* show strain dependent reductions under dry-fermented sausage production and post-processing conditions. Int. J. Food Microbiol. 155, 227-233.

Roig-Sagués, A.X., Hernández-Herrero, M.M., López-Sabater, E.I., Rodríguez-Jerez, J.J., Mora-Ventura, M.T., 1999. Microbiological events during the elaboration of "fuet", a Spanish ripened sausage - Relationships between the development of histidine- and tyrosine-decarboxylase-containing bacteria and pH and water activity. Eur. Food Res. Technol. 209, 108-112.

Sedgwick, G.W., Fenton, T.W., Thompson, J.R., 1991. Effect of protein precipitating agents on the recovery of plasma free amino acids. Can. J. Anim. Sci. 71, 953-957.

Shalaby, A.R., 1996. Significance of biogenic amines to food safety and human health. Food Res.Int. 29, 675-690.

Silla Santos, M.H., 1996. Biogenic amines: their importance in foods. Int. J. Food Microbiol. 29, 213-231.

Sinz, Q., Schwab, W., 2012. Metabolism of amino acids, dipeptides and tetrapeptides by *Lactobacillus sakei*. Food Microbiol. 29, 215-223.

Stahnke, L.H., Holck, A., Jensen, A., Nilsen, A., Zanardi, E., 2002. Maturity acceleration of Italian dried sausage by *Staphylococcus carnosus*-relationship between maturity and flavor compounds. J. Food Sci. 67, 1914-1921.

Su, M.S., Schlicht, S., Gänzle, M.G., 2011. Contribution of glutamate decarboxylase in *Lactobacillus reuteri* to acid resistance and persistence in sourdough fermentation. Microb. Cell Fact. 10, 1-12.

Suzzi, G., Gardini, F., 2003. Biogenic amines in dry fermented sausages: a review. Int. J. Food Microbiol. 88, 41-54.

Teixeira, J.S., Seeras, A., Sanchez-Maldonado, A., Zhang, C., Su, M.S., Gänzle, M.G., 2014. Glutamine, glutamate, and arginine-based acid resistance in Lactobacillus reuteri. Food Microbiol. 42, 172-180.

Toelstede, S., Hofmann, T., 2008. Quantitative studies and taste re-engineering experiments toward the decoding of the nonvolatile sensometabolome of gouda cheese. J. Agric. Food Chem. 56, 5299-5307.

Toldrá, F., Rico, E., Flores, J., 1992. Activities of pork muscle proteases in model cured meat systems. Biochimie. 74, 291-296.

Toldrá, F., 1998. Proteolysis and lipolysis in flavour development of dry-cured meat products. Meat Sci. 49, 101-110.

Toldrá, F., Flores, M., 1998. The role of muscle proteases and lipases in flavor development during the processing of dry-cured ham. Crit. Rev. Food Sci. Nutr. 38, 331-352.

Ueda, Y., Yonemitsu, M., Tsubuku, T., Sakaguchi, M., Miyajima, R., 1997. Flavor characteristics of glutathione in raw and cooked foodstuffs. Biosci. Biotechnol. Biochem. 61, 1977-1980.

Vermeulen, N., Gänzle, M.G., Vogel, R.F.,2007. Glutamine deamidation by cereal-associated lactic acid bacteria. J. Appl. Microbiol. 103, 1197-1205.

Vignolo, G., Fontana, C., Fadda, S., 2010. *Semidry and dry fermented sausages*. In: Toldrá, F. (Ed.), Handbook of Meat Processing. Wiley-Blackwell, Iowa, pp. 379-398.

Whittenbury, R., 1964. Hydrogen peroxide formation and catalase activity in the lactic acid bacteria. J. Gen. Microbiol. 35, 13-26.

Zhao, C.J., Gänzle, M.G., 2016. Synthesis of taste-active γ-glutamyl dipeptides during sourdough fermentation by *Lactobacillus reuteri*. J. Agric. Food Chem. 64, 7561-7568.

Zhao, C.J., Kinner, M., Wismer, W., Gänzle, M., 2015. Effect of glutamate accumulation during sourdough fermentation with *Lactobacillus reuteri* on the taste of bread and sodium-reduced bread. Cereal Chem. 92, 224-230.

Zheng, J., Ruan, L., Sun, M., Gänzle, M.G., 2015. A genomic view of *Lactobacilli* and *Pediococci* demonstrates that phylogeny matches ecology and physiology. Appl. Environ. Microbiol. 81, 7233-7243.

Zuber, A., Horvat, M., 2007. Influence of starter cultures on the free fatty acids during ripening in Tea sausages. Eur. Food Res. Technol. 224, 511-517.

Zúñiga, M., Miralles, M.d.C., Pérez-Martínez, G., 2002. The product of *arcR*, the sixth gene of the arc operon of *Lactobacillus sakei*, is essential for expression of the arginine deiminase pathway. Appl. Environ. Microbiol. 68, 6051-6058.

Appendix

Table S1. Changes of moisture loss and water activity during sausage fermentation.

Moisture loss (%)					
Time (d)	1	3	4	11	20
Aseptic control	1.38	2.90	3.99	16.08	30.83
L. sakei	1.39	2.88	4.49	14.55	35.99
L. plantarum	1.45	2.66	3.36	21.01	38.81
P. pentosaceus	2.07	4.15	6.93	19.66	41.00
P. acidilactici	2.08	4.76	5.87	19.34	39.84
L. sakei and *S. carnosus*	0.96	3.17	4.22	11.11	32.24
P. pentosaceus and *S. carnosus*	1.56	2.96	4.28	9.63	34.78
Water activity					
Time (d)	0	1	3	4	20
Aseptic control	0.94	0.94	0.95	0.94	0.87
L. sakei	0.94	0.94	0.95	0.95	0.87
L. plantarum	0.94	0.94	0.93	0.92	0.88
P. pentosaceus	0.95	0.94	0.94	0.94	0.83
P. acidilactici	0.95	0.94	0.95	0.94	0.86
L. sakei and *S. carnosus*	0.95	0.95	0.95	0.94	0.90
P. pentosaceus and *S. carnosus*	0.94	0.95	0.95	0.94	0.90

Data represent means of triplicate independent fermentations.

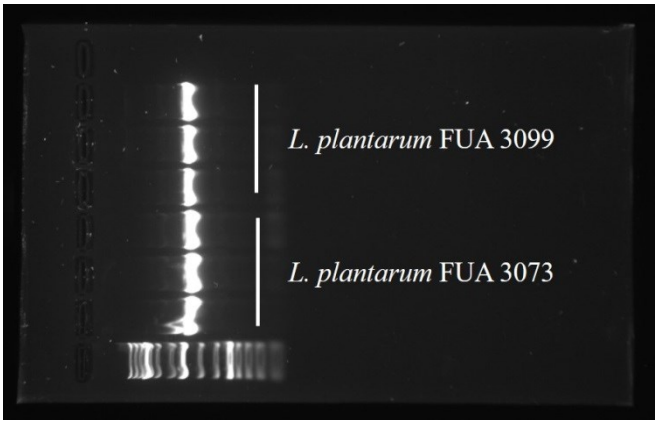

Figure S1. Gel electrophoresis of *gadB* fragment in *L. plantarum* FUA 3073 and FUA 3099.

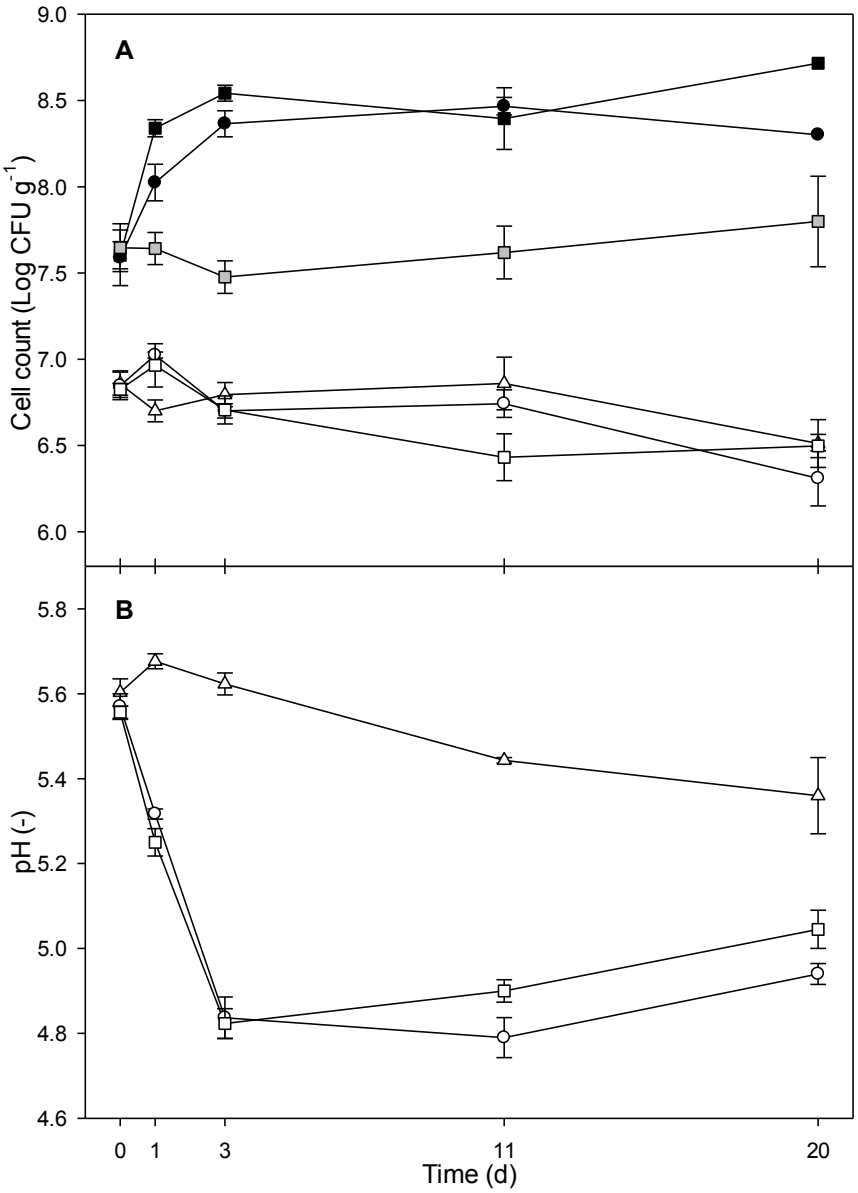

Figure S2. Cell counts **(Panel A)** and pH **(Panel B)** of EHEC positive control and inoculated sausages. Data are presented as means of triplicate biological repeats with the standard error of the mean. **Panel A**, symbols indicate the EHEC cell counts on LB agar in the positive control (△), *L. sakei* sausages (○), *L. sakei* and *S. carnosus* sausages (□); cell counts of *L. sakei* in the single strain fermented sausages (●) and *L. sakei* and *S. carnosus* cocktail fermented sausages (■); cell counts of *S. carnosus* in *L. sakei* and *S. carnosus* cocktail fermented sausages (■). **Panel B**, symbols indicate the pH of the positive control sausages (△), *L. sakei* fermented sausages (○), and *L. sakei* and *S. carnosus* cocktail fermented sausages (□).