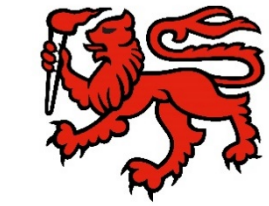

Quantitative Description of Interactions among a Meat Bacterial Spoilage Community

by

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This thesis includes work, which has been published, submitted or to be submitted for publication in a peer-review journal. More details for each paper are described in the section of "Publications Arising from the Thesis". The following people and institutions contributed to the publication of work undertaken as part of this thesis:

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Effect of undissociated lactic acid on growth kinetics and interactions of *Carnobacterium maltaromaticum*, *Brochothrix thermosphacta* and *Serratia liquefaciens* in mixed culture.

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We, the undersigned, endorse the above stated contribution of work undertaken for each of the published (or submitted) peer-reviewed manuscripts contributing to this thesis:

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Table of Contents

List of Tables

List of Figures

Abstract

Spoilage bacteria are a primary factor affecting the shelf-life of vacuum-packaged (VP) beef. Common bacterial species that dominate microbial communities of VP beef include *Carnobacterium maltaromaticum, Brochothrix thermosphacta*, and *Serratia liquefaciens*. However, a quantitative description of how these species establish and dynamically interact within a beef spoilage community, under commercial VP conditions, has yet to be reported.

The pH, lactic acid, and glucose concentrations of beef can markedly impact bacterial growth. The effects of these combined factors on individual growth kinetics of *C. maltaromaticum, B. thermosphacta,* and *S. liquefaciens* were studied in a simulated beef matrix (modified brain heart infusion broth; mBHI broth) within a commercial VP heat-shrunk film. pH (5.5, 6.5), lactic acid (50 mM), and undissociated lactic acid (UDLA; 0.11, 1.12 mM) significantly impacted bacterial growth rate (GR) and maximum population density (MPD). Of the three species, *S. liquefaciens* displayed high growth and tolerance to low pH and high concentrations of lactic acid and UDLA, which was followed by *C. maltaromaticum* and *B. thermosphacta*, the latter being the most sensitive species.

Current culture-based methods are not adequate to measure bacterial growth kinetics in 2- and 3-species experimental systems. Therefore, a SYBR green-based qPCR method targeting the 16S rRNA gene of *C. maltaromaticum, B. thermosphacta* and *S. liquefaciens* was developed. Primers were designed based on 16S rRNA gene sequences. DNA extraction was optimized and a comparatively high annealing temperature (65^{o}C) used. The reaction efficiency of standard curves was high (R^2 = 0.98-0.99) over a linear quantification range of >5 log CFU/ml. Coefficient of variation did not exceed 14% within or between runs. The calculated GR and MPD were not significantly different between plate count and qPCR

methods. Validation in mixed culture showed a variance of less than 0.3 log CFU/ml ($\bar{x}$ = 0.10 log CFU/ml, R^2 = 0.98).

The qPCR method was then implemented to study the effect of UDLA on bacterial growth kinetics in 1-, 2- and 3-species experimental systems. mBHI broth was formulated with a range of lactic acid concentration (0, 25, 50, 75, 100 mM) and pH (5.5, 6.5) and packaged within a commercial VP film, which was then heat-shrunk following a commercial protocol to reduce oxygen permeability. The highest species sensitivity to 0 to 2.24 mM UDLA occurred in the order: *B. thermosphacta* > *C. maltaromaticum* > *S. liquefaciens*. No growth was observed for any species at 2.24 mM UDLA.

Interaction among the species was evaluated by subtracting the GR and MPD observed in the 3-species mixed culture from that of the pure culture. For *B. thermosphacta*, GR and MPD were significantly inhibited in the 3-species culture at 0.56 and 1.12 mM, and from 0.06 to 1.12 mM UDLA, respectively. A trend in increase of MPD difference between individual and 3-species mixed culture was observed up to 0.22 mM UDLA. Evaluation of 2-species interactions revealed *C. maltaromaticum* had the greatest inhibitory effect on *B. thermosphacta* growth and reducing GR and MPD at 1.12 mM UDLA to 0.001 CFU/h and 3.20 CFU/ml, respectively. Unlike *B. thermosphacta*, GR of *C. maltaromaticum* was increasingly greater in the 3-species versus pure culture from 0.56 to 1.68 mM UDLA; MPD was increasingly lower in 3-species culture from 0.06 to 0.22 mM UDLA, but not at 0.56 and 1.12 mM UDLA. Unexpectedly, *C. maltaromaticum* grew at 1.68 mM UDLA in the 3-species mixture but not in pure culture, an effect not attributed to a change in pH during culture. The effector specie(s) causing *C. maltaromaticum* growth inhibition or promotion was not observed in 2-species culture, indicating the effect required a 3-species interaction. UDLA

had no effect on *S. liquefaciens* GR or MPD in 3-species mixed culture. The 'Jameson effect' may have caused the reduction in *B. thermosphacta* MPD in 2-species co-culture with *C. maltaromaticum*, however the inhibitory effect produced by *S. liquefaciens* was likely caused by a different mechanism. Individual bacterial growth kinetics in mBHI broth were validated in irradiated VP beef, demonstrating an acceptable accuracy factor of 1.12 for all three species. In conclusion, this thesis provides the beef industry with science-based evidence to more effectively design intervention strategies to control the evolution of beef spoilage microbiomes.

Chapter 1. General introduction

1.1 Background and study aim

Australia is the world's third largest beef exporter, shipping 72% of total production to more than 70 countries, with an associated total value of A$9.5 billion in 2018 - 2019 (MLA, 2019). Maintenance of beef shelf-life during shipping to international markets is essential. Unavoidable microbial contamination during slaughter and carcass processing, and subsequent microbial growth, results in beef spoilage that if not sufficiently constrained, leads to economic loss for meat industries (Bell and Garout, 1994; Nattress et al., 2001; Odeyemi et al., 2020).

Extrinsic (atmospheric condition, temperature) and intrinsic factors (initial microbial load, fat content, pH, lactate or lactic acid, water activity, glucose) are potential factors that influence bacterial growth and beef spoilage (Iulietto et al., 2015; Nychas et al., 2008). To attain extended shelf-life and limit microbial spoilage, vacuum-packaging (VP) and storage at low temperature are widely employed primary strategies (Nychas et al., 2008; Sun and Holley, 2012). Apart from these approaches, quantitative knowledge is required to understand spoilage bacterial behaviour in response to other potential inter-related factors, e.g. glucose content, pH, and lactic acid. These factors are relevant, since key SSOs contaminating meat can metabolise glucose as a primary carbon source and produce lactic acid by fermentation, which in turn reduces meat pH (Gill, 1986).

The development and diversity of the bacterial spoilage microbiome in VP beef is affected by inter- and/or intra-species interactions (Bili et al., 2016; Zhang et al., 2015). This includes inhibitory effects of one strain of *Carnobacterium maltaromaticum* on a second *C.*

maltaromaticum strain (Zhang et al., 2017). Consequently, more fundamental and mechanistic insights about spoilage community formation are needed, especially for mixed-culture systems to quantitively describe intra- and inter-species interactions.

Conventional methods to cultivate bacteria in bacteriological media are considered the "gold standard" for growth kinetic studies, however they are often insufficient for measuring growth kinetics of single strains within mixed cultures, even using selective or differential media (Clais et al., 2015; Li and Chen, 2013; Urbán et al., 2010). Additionally, culture-based methods are time and labour intensive with low sensitivity and specificity (Liu et al., 2019). In contrast, molecular methods such as quantitative polymerase chain reaction (qPCR) are highly sensitive, specific and rapid, providing accurate quantification of single strains within high concentrations of background microorganisms. Yet, optimisation of each step in qPCR is required prior to implementing a validated technique to investigate the effects of environmental factors on multi-species bacterial interactions (Ceuppens et al., 2014; Liu et al., 2019; Postollec et al., 2011).

Lactic acid is "GRAS" (generally recognised as safe) and a biological component of beef. It is well known to restrict bacterial growth, especially in the undissociated form (UDLA), which can reduce bacterial growth by altering cell metabolism (Greer and Dilts, 1995; Janssen et al., 2007; Russell, 1992; Shelef, 1994). The concentration of UDLA is strongly influenced by the interaction of pH and lactic acid. As of this date, there are no known reports about the effects of pH, lactic acid (and UDLA) on bacterial interactions in mixed-culture systems, and the impact on beef microbiomes.

To solve these knowledge gaps, this study

Investigated the effect of pH, lactic acid (and UDLA), and glucose on pure cultures of *C. maltaromaticum, B. thermosphacta* and *S. liquefaciens* within a commercial heat-shrunk VP film,

Developed a qPCR method to measure growth kinetics of *C. maltaromaticum, B. thermosphacta* and *S. liquefaciens* in a mixed-culture broth system, and

Analysed the effect of UDLA on growth kinetics of and interactions among *C. maltaromaticum, B. thermosphacta* and *S. liquefaciens* in mixed-culture systems.

1.2 Structure of the thesis

This thesis contains six chapters:

Chapter 1: Background, aims, and structure of the thesis are described (current chapter).

Chapter 2: Background of current research regarding general aspects of beef spoilage, dominant bacterial species on VP beef, and the effect of potential environmental factors on bacterial growth and interactions.

Chapter 3: Effect of glucose, pH and lactic acid on *Carnobacterium maltaromaticum, Brochothrix thermosphacta* and *Serratia liquefaciens* within a commercial heat-shrunk vacuum-package film was studied. This chapter has been published in the journal *Food Microbiology*.

Chapter 4: qPCR quantification of *Carnobacterium maltaromaticum, Brochothrix thermosphacta*, and *Serratia liquefaciens* growth kinetics in mixed culture. This chapter has been published in the *Journal of Microbiological Methods.*

Chapter 5: Effect of undissociated lactic acid on growth kinetics and interactions of *Carnobacterium maltaromaticum*, *Brochothrix thermosphacta* and *Serratia liquefaciens* in mixed culture, are described. This chapter is being developed for submission to a peer-reviewed journal.

Chapter 6: The results achieved in this thesis and future research directions are discussed.

Chapter 2. Literature review

2.1 Introduction

Spoilage of fresh beef is the manifestation of microbial growth and metabolism, which can be detected as a formation of off-flavours, slime formation, discolouration, gas production or any other unusual textural appearances that makes the meat unsuitable for human consumption (Gram et al., 2002; Jackson et al., 1992). Due to high water content and significant concentrations of nutrients, muscle tissue is highly susceptible to microbial growth (Sun and Holley, 2012).

The storage life of meat products is usually the time at which the product can be stored before these signs of spoilage become overt. The storage-life of Australian boneless beef is no less than 12 weeks to 26 weeks under optimum storage specifications (Small et al., 2012). Such storage specifications are achieved by using secure vacuum-packaging (VP) films, along with storage at -1^{o}C, while maintaining temperature through the cold chain from processor to retailer (MLA, 2014).

Prior to VP, initial microbial contamination of beef occurs while slaughtering, skinning, cutting, and processing. At the initial stage, Specific Spoilage Organisms (SSO) form a minor part of the total microbial community (Huis in't Veld, 1996). The growth of SSO to unacceptable levels, and their production of metabolites, influences the sensory spoilage pattern (Jaaskelainen et al., 2016). *Pseudomonas, Acinetobacter/Moraxella (Psychrobacter), Shewanella putrefaciens, Clostridium*, Lactic Acid Bacteria (LAB), *Enterobacteriaceae, and Brochothrix thermosphacta* are members of spoilage-associated microbial community, common in refrigerated beef (Borch et al., 1996).

During storage, interactions among bacteria may play a major role in determining the growth and composition of the spoilage community (Mangano et al., 2009; Skandamis and Nychas, 2012). It is common for bacteria within a community to interact by producing bacteriocins, organic acids, auto-inducers (quorum-sensing factors), enzymes and/or intercellular structures (e.g. in cell contact-dependent communication) (Allende et al., 2007; Cintas et al., 1998; Schöbitz et al., 2006). Interactions can also be regulated by intrinsic (pH, water activity, metabolites) and extrinsic (temperature, atmosphere) factors (Buchanan and Bagi, 1997; Koutsoumanis et al., 2006).

This review describes issues that impact the Australian beef industry, aspects of beef spoilage and factors influencing microbial growth, including the dominant microflora of VP beef, and the role of interactions among bacteria and factors regulating bacterial interaction.

2.2 Beef production and export in Australia

Being one of the leading suppliers of high-quality beef, Australia exports chilled and frozen meat products which helps to meet the requirement for red meat protein around the world (MLA, 2015). Although it produces just 3.9% of the world's total beef, over 64% worth, $6.3 billion is exported, making Australia the world's third largest beef exporter, behind Brazil and India (MLA, 2019). In the area of food safety, product stability, and identification, the Australian meat and livestock industry holds a remarkable responsibility and its product quality is a perfect companion to Australia's emphasis on responsibility in dealing with the demands of international customers (MLA, 2013).

Without proper handling of raw meat, 40% of the total production could be spoiled, represents a significant loss for the meat industry, retailers and consumers (Sun and Holley, 2012). Although a number of new packaging strategies have been developed to improve supply chain performance, the Australian beef industry relies on VP technology as a mechanism to extend meat shelf-life during long periods of shipment and storage (i.e. intercontinental transport) (Rodas-Gonzalez et al., 2011).

As beef provides nutrients needed to support the growth of many types of microorganisms, it ultimately spoils as a consequence of such growth, unless chilled to low temperature (e.g. -1^{o}C) and packaged in a controlled atmosphere (Bell and Garout, 1994; Egan et al., 1988). Appropriate preservation methods can be employed to increase shelf-life of fresh raw meat by ensuring controlling temperature, packaging atmosphere, water activity, and/or using chemical or bio-preservatives (Zhou et al., 2010). However, more efforts and considerations are required to design an effective approach to preserve beef prior to spoilage.

2.3 General aspects of beef spoilage

Spoilage can be defined as the production of volatile organic compounds with off-flavours, and off-odours that make meat offensive and undesirable for consumer consumption (Dainty, 1996; Gram et al., 2002; Jackson et al., 1992). It is evident from previous research that when SSO colonise meat and grow to high levels, spoilage occurs (La Storia et al., 2012; Nychas et al., 2008). Initially, the bacterial level of meat and meat products is relatively low (e.g. 10^2-10^3 CFU/cm^2), consisting of a wide variety of species (Blickstad et al., 1981; Blickstad and Molin, 1983; Blixt and Borch, 2002; Jackson et al., 1992); 10% of the initial microbial community may constitute SSOs (Borch et al., 1996; Huis in't Veld, 1996). Throughout storage, levels of bacteria increase exponentially after an initial lag, reaching

10^7 - 10^8 CFU/cm^2, and producing mildly offensive, acidic, 'dairy spoilage' odours and flavours. These detectable changes occur only after the high numbers have been attained (Borch et al., 1996; Sofos, 1994).

The evolution of organoleptic spoilage is related to microbial consumption of meat nutrients, for instance glucose, free amino acids, lactic acid, urea, and hydrophilic protein, resulting in the release of volatile compounds as well as metabolites, aldehydes, esters, ketones alcohols, amines, organic acids, and sulphur compounds. All of these compounds determine the sensory characteristics of meat spoilage (Casaburi et al., 2015; Dainty et al., 1989a; Ercolini et al., 2009; Gill, 1983; Jaaskelainen et al., 2016; Kakouri and Nychas, 1994; Lambert et al., 1991; McMeekin, 1982; Nychas et al., 2007; Youssef et al., 2014a).

The function of microbial enzymes in the spoilage process is still ambiguous and Nychas et al. (2008) minimized the role of these enzyme prior to accumulation of metabolic by-products. The production of exoprotease by Pseudomonads helps them to help it penetrate the meat matrix and utilize sources of nutrition for their growth (Garcia-Lopez et al., 1998; Gill and Penney, 1977). Likewise, although most psychotrophic bacteria produce lipases, although the specific function of lipase producing bacteria during the occurrence of meat spoilage through lipolytic and oxidative changes is not well established (Garcia-Lopez et al., 1998).

Microbial activity is not the only reason for meat spoilage. Spoilage can also take place biochemically by meat lipid oxidation. Oxidative degradation and production of off-flavour is the consequence of autoxidation of meat lipids. During this biochemical change, free radicals are produced which alter meat fatty acids (Dave and Ghaly, 2011). Morrissey et al. (1998) found that, along with discoloration, off-odour, drip loss, and off-taste, lipid

oxidation may also produce potentially toxic compounds even in the absence of microorganisms.

Enzymatic hydrolysis of lipid involves certain types of endogenous meat enzymes such as lipase, esterase, and phospholipase. The chemical reaction of lipolysis involves break down of glycerides producing free fatty acids leading to off-odours, commonly known as rancidity (Huis in't Veld, 1996). Haemoglobin, myoglobin and cytochrome are heme proteins of meat and are also susceptible to oxidation producing hydro-peroxides (Kanner, 1994).

In the muscle cells of slaughtered animals, enzyme activity occurs naturally and is one of the leading causes of meat deterioration (Dave and Ghaly, 2011; Tauro, 1986). The ultimate result of autolysis processes can change meat colour (e.g. greenish) and can soften meat by breaking down complex tissue compounds (e.g. carbohydrates, fats and protein) into simpler metabolites (Dave and Ghaly, 2011). Generally, autolytic changes consist of proteolysis and fat hydrolysis, producing potential by-products which are regulatory factors for microbial decomposition (Tauro, 1986). Extensive autolysis is termed as “souring”, caused by calpains, cathepsins and aminopeptidases. During post-mortem autolysis, myofibril proteins (z-line) are digested and autolysis of meat or muscle cells take place by these indigenous enzymes (Huss, 1995; O'Halloran et al., 1997). Although calpains and cathepsins are primary contributors to proteolytic meat tenderization, calpains play a major role in catalysing reaction of meat proteolysis. Kuwahara and Osako (2003), found that even at low temperature (5°C), protein degrading enzymes are active, causing meat quality deterioration by promoting microbial growth as well as biogenic amine production.

2.4 Factors influencing microbial growth on beef and beef products

Within production and trade of meat, each step can introduce different environmental conditions creating specific ecological niches that support contaminating microbial strains (Castellano et al., 2008; Nychas et al., 2008). However, there are some potential factors that influence the shelf-life of meat products by favouring certain bacterial species over others; these include: packaging (aerobic, vacuum, and modified atmosphere), composition of beef products (fat, nitrites, NaCl content, pH, aw,), storage temperature, and chemicals used for preservations such as bio-preservatives or antibacterial substances (Nychas et al., 2008; Remenant et al., 2015). Indeed, storage temperature and atmosphere are major factors influencing microbial associations with beef, as well as the deterioration process (Ercolini et al., 2010; Nychas et al., 2008; Pennacchia et al., 2011; Stanbridge and Davies, 1998). In addition, initial microbial contamination level has been identified as a potential factor in growth of spoilage related microorganisms of red meat. Application of a time-temperature chilling profile could be beneficial to predict and retard such bacterial growth which is dependent on initial contamination level (EFSA BIOHAZ panel, 2016).

2.4.1 Extrinsic factors

Packaging atmosphere

The composition of spoilage flora is greatly influenced by packaging condition and the atmospheric gaseous composition surrounding meat (Borch et al., 1996; Iulietto et al., 2015). Depending on meat cut and expected storage time, three common types of packaging, modified atmosphere package (MAP), vacuum package (VP) and aerobic package (AP), are commonly used by meat industries, processors and retailers. In Australia, VP is

commonly used with boneless primal cuts of lamb and beef, for ease in handling, to restore meat colour, and to reduce the growth of spoilage bacteria (CSIRO, 2009).

Aerobic packaging stimulates growth of Pseudomonads, whereas VP or CO_2 MAP shifts microbial flora to facultative anaerobic species, such as lactic acid bacteria (LAB) and *Brochothrix thermosphacta* (Nychas et al., 2009; Pennacchia et al., 2011; Stanbridge and Davies, 1998).

Temperature

It is well known that the bacterial lag time, growth rate, and maximum density are influenced by storage temperature (Nychas et al., 1998). Lower refrigeration temperature reduces growth rate and modifies the structure of spoilage microbial population that is already exist in meat (Nychas et al., 2008). However, at lower temperatures, spoilage of meat can still occur after extended periods of storage as the growth pattern is highly variable. For example, *Carnobacterium* spp. prevail in VP beef at -1.5°C, and 4°C and 7°C allow homo-fermentative *Lactobacillus* spp. to dominate (Ray, 2013). In the group of Enterobacteriaceae, *Hafnia alvei* can dominate at 1.5°C and 4°C, respectively (Borch et al., 1996; Iulietto et al., 2015). In addition, psychrophilic *Clostridium* spp. was also identified in VP chilled meat (Broda et al., 2002; Dainty et al., 1989a). Temperature abuse can even cause pathogenic species to grow and produce toxic compounds when the product remains at favourable temperatures for a significant period of time of growth (Cenci-Goga et al., 2014; Cenci-Goga et al., 2005; Leonard, 2011).

<u>Meat chilling and transport</u>

Within the processing plant, the meat chill chain comprises two main steps: primary and secondary chilling. Primary chilling occurs immediately after slaughter, when animal body

temperature is reduced to refrigeration temperature in a chiller cabinet. During this time period, spoilage microorganisms grow. Following primary chilling, handling and processing, for example cutting and mincing can increase the temperature of meat, and thus the temperature needs to maintain below 7^{o}C through secondary chilling is required. To attain microbiological balance, production output and consumer acceptability, both chilling steps are highly required (Koutsoumanis et al., 2005).

From production to distribution and marketing to the final user, home refrigerators, retail cabinets and trucks are used to store and transport meat and meat products. These supply chain stops require strict maintenance of temperature in order to control the safety and quality of meat (Nychas et al., 2008).

2.4.2 Intrinsic factors

Beef composition

The chemical composition of beef presents a natural ecosystem for micro-organisms, providing sources of energy that support metabolism and growth. However, in spite of richness in vitamins, protein, minerals and lipids, meat is low in carbohydrates. Interestingly, this content contributes to a selective pressure that influences the growth of certain species versus other species, based on different nutrient requirements and metabolic pathways (Doulgeraki et al., 2012; Iulietto et al., 2015; Ray, 2013). For instance, Pseudomonads can use a large array of iron sources required for aerobic metabolism, and become dominant flora (Labadie, 1999). It is also well established by Grau (1980) that combinations of certain chemical and biochemical parameters of meat facilitates the growth of *B. thermosphacta*.

pH

pH is an important intrinsic factor that regulates the growth of spoilage bacteria in beef. Generally, the pH of muscle reduces to 5.4-5.8 during post slaughtering, although the meat of stressed animals is normally an undesirable level of >6 (Aymerich et al., 2002; Blixt and Borch, 2002). LAB, which produce lactic acid, have a major effect in lowering pH in VP and MAP beef (Sun and Holley, 2012). In some research article it was concluded that the growth of *Pseudomonas* was inhibited by low pH produced by LAB (Blixt and Borch, 2002; Koutsoumanis et al., 2006), while in other research the growth kinetics of *Pseudomonas* spp. did not change in the pH range of 5.3 to 7.8 (McMeekin and Ross, 1996).

Lactic acid

Previous researchers reported that, inherent organic acid of beef muscle acts as bactericidal or bacteriostatic compound against several groups of spoilage organisms (Gill and Newton, 1982; Grau, 1980, 1981). Such inhibitory activity of organic acids is observed in undissociated form when the acids are biologically active at lower pH (Gill, 1986). In addition, apart from inherent organic acids, use of additional acids e.g. citric, acetic at a specific concentration in marinades have been found to regulate the growth of *Salmonella* inoculum and spoilage organisms (Lytou et al., 2019). Post rigor beef muscle contains 0.9-1% lactic acid as a natural constituent. Antibacterial activity of 3% lactic acid solution has been reported against potential pathogenic and spoilage organism including *Pseudomonas fragi, Brochothrix thermosphacta, Listeria monocytogenes, Escherichia coli and Salmonella* spp. (Greer and Dilts, 1995; Dixon et al., 1991; Nattres et al., 1998; Young and Foegeding, 1993). Being a natural element of beef muscle, lactic acid is known as GRAS (generally regarded as safe). While the use of chemical preservatives to extend VP beef shelf life is not acceptable to customers, administration of lactic acid could be an alternative strategy to

eliminate spoilage bacterial growth to extend VP beef shelf life. Effectiveness of lactic acid against spoilage organism is pH dependent. Lower pH produces higher amount of undissociation of lactic acid, such form is reported as an effective inhibitor along with packaging condition (Gill and Newton, 1982; Grau, 1980).

Carbohydrate contents (Glucose, Glucose-6- phosphate, Glycogen)

Initially contaminating microorganisms utilise available low molecular weight substrates (glucose, glucose-6-phosphate and glycogen) for survival and growth (Gill, 1986). Beef muscle contains 0.1, 0.2 and 1% glucose, glucose-6-phosphate and glycogen, respectively. These carbohydrate contents are metabolised into lactic acid by gram positive bacterial species (i.e. LAB and *Brochothrix thermosphacta*) which causes muscle pH to drop (Gill, 1986; Nychas et al., 1988). Bacterial growth is affected to pH fall, however, at this stage

Pseudomonads utilise amino acids which makes spoilage apparent due to production of off-odours (Gill, 1986). Addition of glucose in VP beef was hypothesised by Newton and Gill, (1980) for proliferation of *Lactobacillus* flora to restrict the early spoilage caused by Pseudomonads by organic acid production. Labadie (1999) also reported that meat glucose favours the growth of *Lactobacillus sakei* during the production of ATP, by utilizing arginine.

Water activity (a_w)

The water in food available to support the growth of microorganisms is termed 'water activity' (a_w). Raw meat can support the growth of most microorganisms as it has a_w values of 0.98-0.99 (Aymerich et al., 2002; Garriga et al., 2002). Water activity is crucial to spoilage microorganisms for carrying-out metabolic activities such as enzymatic reactions, synthesizing cellular materials, and conducting other biochemical reactions (Iulietto et al., 2015).

2.5 VP to extend beef shelf-life

Typically, VP is practised by enclosing the beef product in a plastic bag or pouch, which ensures very low moisture and oxygen transmission rates (Scetar et al., 2010). The process is accomplished by heat-sealing of the plastic bags prior to remove air from the package via a vacuum chamber (Lambert et al., 1991). When sealed by a satisfactory vacuum, the amount of O_2 and CO_2 at the package headspace is <1% and 10-20% (v/v) respectively (Lambert et al., 1991). In recent years, meat industries perform heat shrinking treatment following VP which offers several operational advantages, i. e. ease of handling, improving appearance, shelf-life, leak rate, and drip loss (Bell et al., 2001). In addition, heat shrinkage of VP pouches increases thickness reducing the oxygen transmission rate. Such treatment restricts invasion of atmospheric oxygen inside VP pouches which affects the growth of spoilage organisms already present inside VP beef (Newton and Rigg, 1979). The industrial goal of VP is to maintain of muscle colour and fat appearance, slow bacterial growth and thus accumulation off odours. In comparison with beef preserved in CO_2, VP with heat shrinkage provides more advantages like less trimming, and much longer display life (Scetar et al., 2010). By removal and restriction of oxygen, the growth of typical aerobic spoilage microorganisms is reduced, and so is widely used for distribution to retailers of dry-cured products and packaging of primal cuts (Labadie, 1999; Parra et al., 2010; Rubio et al., 2006; Venter et al., 2006).

2.6 Spoilage-related bacteria

Psychotropic bacteria that include *Acinetobacter*, *Pseudomonas*, *Brochothrix*, *Flavobacterium*, *Psychrobacter*, *Moraxella*, *Staphylococcus*, *Micrococcus*, *Clostridium*, LAB and different genera of the family of Enterobacteriaceae are able to grow in meat at chill temperatures are (Dainty et al., 1986; Dainty and Mackey, 1992; Doulgeraki et al., 2012;

Labadie, 1999). However, psychrotrophic LAB are characterized as predominant microflora in most cases of VP-chilled beef (Borch et al., 1996; Dainty et al., 1982; Hitchener et al., 1982; Nychas et al., 1998; Shaw and Harding, 1989; Shaw and Harding, 1984).

It is evident from previous research articles that, although a large variety of microorganisms persist in beef, only a few species dominate to cause spoilage, because temperature, time of storage and packaging atmosphere affect both microbial growth and species selection during fresh meat storage (Doulgeraki et al., 2010; Doulgeraki et al., 2011; Ercolini et al., 2010; Ercolini et al., 2011; Ercolini et al., 2009; Ercolini et al., 2006; Pennacchia et al., 2011).

2.6.1 LAB

The most commonly isolated bacteria from VP beef are psychrotrophic LAB, which are able to grow at chilled temperature, under partial oxygen and carbon dioxide pressure (Borch et al., 1996). A review study stated that the number of LAB is low at the initial time of storage (1-2 log/cm^2)) but increases (7- 8 log/cm ^{2}) within 6-8 weeks, due to adaptive mechanisms under VP storage conditions (Hernandez-Macedo et al., 2011; Jones, 2004). It has been observed that under vacuum, LAB reached densities as high as 10^7 CFU/cm^2 (Gill and Newton, 1978).

LAB consists of *Lactobacillus*, *Leuconostoc*, *Enterococcus*, *Lactococcus*, and *Carnobacterium* genera (Brightwell et al., 2009; Chaves et al., 2012; Gill and Badoni, 2002; Jones, 2004; Laursen et al., 2005; Pennacchia et al., 2011; Sakala et al., 2002; Samuel et al., 2011; Yost and Nattress, 2002; Youssef et al., 2014a). Among these taxa, the species *Carnobacterium maltaromaticum* and *Carnobacterium divergens* are commonly found on VP beef (Casaburi et al., 2015; Ercolini et al., 2010; Jones, 2004; Laursen et al., 2005; Pennacchia et al., 2011; Sakala et al., 2002; Stanbridge and Davies, 1998).

Heterofermentative LAB, such as *Carnobacterium* spp., can ferment glucose and other substrates of meat, and then produce lactic acid as well as CO_2, which is the main reason for presence of liquid in package and accumulation of gas leading to package failure (Hernandez-Macedo et al., 2012; Jones, 2004). Along with CO_2, LAB can occasionally produce H_2S from cysteine that produces metmyoglobin by oxidizing myoglobin which change the colour of meat into a greenish one and unpleasant odour (Hernandez-Macedo et al., 2011). It was also found that metabolites such as acetoin, 1-octen-3-ol, butanoic acid, aldehydes, lactones, and sulphur-containing compounds, can be produced by *Carnobacterium maltaromaticum*, which leads beef to deterioration (Ercolini et al., 2009).

The metabolic residues of lactic acid can be assessed as milky or slightly acidic tastes. The off-odours of volatile fatty acids produced by *Lactobacillus curvatus* and *L. sakei* usually disappears after opening the bag. So, this kind of deterioration is not treated as particularly unacceptable (Hernandez-Macedo et al., 2011). However, a few LAB species contribute to meat spoilage via these metabolites, leading to organoleptic deterioration of meat (Pothakos et al., 2015).

In spite of regarding LAB as a spoilage organism, they have been recommended as protective cultures to inhibit the growth of other spoilage organism, by producing organic acid and antimicrobial compounds (Hugas, 1998; Pothakos et al., 2015; Signorini et al., 2006). In several studies it was proven that, *L. sakei* delay the spoilage of VP beef from blown pack spoilage, by reducing the total spoilage microbial counts (Jones et al., 2009; Katikou et al., 2005). Similar finding was also reported by Rahkila et al. 2012, who found artificial inoculation of lactococci in high concentration restricted the growth of naturally occurring spoilage related LAB without being dominant in MAP meat products. Ribotyping

analysis of *L. sakei* isolated from a spoiled meat product processed in fermented product handling room also confirmed that this strain was not dominant to cause the spoilage (Bjorkroth and Korkeala, 1996).

2.6.2 Brochothrix thermosphacta

B. thermosphacta is a Gram-positive, non-spore forming, homofermentative, facultatively anaerobic rod-shaped bacterium, which is an important microorganism in spoilage of refrigerated meat (Pin et al., 2002). It can utilize glucose as a substrate and mainly produces L-(+)-lactic acid, small amounts of short chain fatty acids and ethanol that cause 'sweaty socks' and off-odours under anaerobic condition (Casaburi et al., 2014; Casaburi et al., 2011; Holm et al., 2013; Holm et al., 2012). Aerobically it produces di-acetyl and acetoin causing 'sweat' odours that are normally present in some cheeses (Pin et al., 2002).

B. thermosphacta can produce histamine, putrescine, tyramine, cadaverine and tryptamine in meats stored in the presence of oxygen (Emborg et al., 2005; Nowak and Czyzowska, 2011; Papadopoulou et al., 2012). It was concluded by researchers that the aerobic metabolism of this bacterium is more offensive and stronger than anaerobic metabolism (Kameník et al., 2014; Pennacchia et al., 2011; Sakala et al., 2002). Although it is well established that LAB can produce lactic acid and inhibit growth of *B. thermosphacta*, VP is also regarded as a method to reduce the viable number *B. thermosphacta* which could compete against LAB in chilled meat (Grau, 1980; Newton and Gill, 1978; Russo et al., 2006).

Organoleptic changes of meat usually take place by lipolytic and proteolytic activities by this particular species of bacteria but it depends on the strains as well as temperature. Researchers found that a number of *B. thermosphacta* have lipolytic activity at higher temperatures by synthesizing lipase such as at 20 and 25^{o}C (Braun and Sutherland, 2003;

Nowak et al., 2012; Papon an Talon, 1988) whereas Casaburi et al. (2014), found none of the strains was able to produce lipase or digest proteins in laboratory conditions at 4^{o}C to 20^{o}C. Likewise, some strains could not degrade proteins or produce exoprotease enzymes below 6^{o}C (Braun and Sutherland, 2003; Labadie, 1999). Considering these studies, it can be hypothesised that, along with strains, temperature also plays an important role for the influence of lipolytic or proteolytic activity.

2.6.3 Enterobacteriaceae

Cold-tolerant *Enterobacteriaceae* play significant roles in rapid proliferation and acceleration of spoilage if meat is stored at >4^{o}C and pH above 5.8, under anaerobic condition (Sakala et al., 2002; Youssef et al., 2014a; Youssef et al., 2014b). Fresh beef normally contains of a small population of *Enterobacteriaceae* that multiply in VP condition, causing pack distension and deterioration at refrigeration temperature (Degirmencioglu et al., 2012).

Serratia spp., *Hafnia alvei, Rahnella spp*., and *Yersinia enterocolitica* are the most frequently occurring genera detected on VP as well as MAP beef, and other species occasionally, such as *Citrobacter freundi*, can also be isolated (Brightwell et al., 2009; Ercolini et al., 2009; Yost and Nattress, 2002, Sade et al., 2012). *Hafnia and Serratia* have been reported to produce diamine and cause meat spoilage (Dainty et al., 1986; Edwards et al., 1985; Gill and Penney, 1988). It is established from a research work that some species of *Enterobacteriaceae*, for instance, *Serratia*, *Enterobacter, Hafnia, Ewingella, Rahnella* were the causative agent of blown pack spoilage which was characterized by gas production and pack distension at 4°C (Brightwell et al., 2007). Facultative anaerobes particularly*, Serratia*, *Enterobacter*, *Hafnia,*

Proteus utilize amino acids by producing ammonia, amines, dimethyl sulphide and mercaptans that ultimately cause putrefaction (Ray and Bhunia, 2007).

2.6.4 Clostridium spp.

Psychrotrophic and psychrophilic *Clostridium* may cause deep muscle anaerobic spoilage of VP meats. In addition, they are treated as a causative agents of blown VP product (Broda et al., 1996). Proteolysis is the principle biochemical process adopted by *Clostridium* for worsening spoilage such as changes in physical appearance, distinguishing odour (due to the production of H_2S) and presence of liquid inside the package (Ray, 2013). In the absence of oxygen, sulphur compounds are produced through the breakdown of proteins which results in offensive and strong odours. In addition, the breakdown of non-protein nitrogen compounds usually produces ammonia (Ray, 2013).

Clostridium algidixylanolyticum, C. algidicarnis, C. frigidicarnis, C. estertheticum, C. gasigenes, and C. putrefaciens are the dominant species of cold tolerant Clostridia that have been found as an elementary agent of blown pack and premature deterioration of VP chilled meat. This pattern of spoilage is detected by gas formation, exudates, off-odours, pH change and change in meat colour, proteolysis within few weeks of storage (Adam et al., 2011; Adam et al., 2010; Broda et al., 2002; Silva et al., 2011).

2.6.5 Pseudomonads

Pseudomonas spp. are the most common spoilage bacteria on fresh meat stored aerobically (Ercolini et al., 2007; Labadie, 1999; Stanbridge and Davies, 1998; Sun and Holley, 2012). According to a review by Labadie (1999), *P. fragi, P. fluorescens and P. putrefaciens* are the species most frequently isolated from meat, and they consume available glucose of meat. Once glucose and lactate of meat are exhausted, amino acids are metabolized, generating

ammonia, amines, organic sulphides and leading to the production of off-odours (Sun and Holley, 2012).

In addition, high amounts of alcohols and ketones are detected in the head space of VP beef samples inoculated with *P. fragi* (Ercolini et al., 2009). *P. putida* is another species that has been frequently reported on beef products with the potential to produce cadaverine, a biogenic amine formed by decarboxylation of lysine, and is associated with meat organoleptic changes and general decay processes (Ozogul and Ozogul, 2007). In anaerobic condition (<1% of oxygen) Pseudomonads are supressed but can still be detected in VP product at lower levels and have spoilage activity by synthesizing many volatile organic compounds (Ercolini et al., 2010; Ercolini et al., 2009; Pennacchia et al., 2011). Mohareb et al. (2015) investigated and confirmed the effect of glucose concentration and temperature on expression of spoilage biomarker genes in *P. putida*. Such report will be beneficial to block or revert the activation pathway of spoilage genes to restrict meat spoilage in future.

2.6.6 Other species

In addition to the bacteria described above, different species of *Acinetobacter, Staphylococcus, Shewanella*, and *Bacillus* have also been isolated during the early stages of VP beef storage (Doulgeraki et al., 2012; Ercolini et al., 2009; Sakala et al., 2002; Youssef et al., 2014a). In a review article of Hernandez-Macedo et al. (2011) it is stated that, *Shewanella putrefaciens* grew and utilized cysteine, and produced hydrogen sulphide and organic sulphides, which have unpleasant odours and react with myoglobin to cause greening of meat. Apart from these other genera, such as *Microbacterium, Flavobacterium, Moraxella, Ralstonia, Limnobacter, and Photobacerium* can also occur on VP beef (Doulgeraki et al., 2012; Ercolini et al., 2011; Pennacchia et al., 2011; Youssef et al., 2014a).

2.7 Interactive behaviour of bacteria during beef spoilage

Previously, spoilage was regarded as an outcome of unlimited growth of spoilage organisms (spoilage association) considering both those which contributed to spoilage and which were present but did not have any contribution to unpleasant deterioration (Nychas et al., 2008). A single species of organism causing specific spoilage was termed as SSO and their potentiality was defined by particular spoilage metabolite production. Later the term 'Metabiotic spoilage association' was introduced by Jorgensen et al. (2000), describing the process of spoilage involving two or more microorganisms exchanging nutrients to produce spoilage metabolites. Thus, the concept of specific spoilage organism was extended to define a set of organisms spoiling a product by interacting among themselves.

Consequence of environmental conditions and microbial interactions influence spoilage process (Nychas et al., 1998; Tsigarida et al., 2003). Competition and cooperation are two main forms of interactions in bacteria (Griffin et al., 2004). Food industries employ positive responses or cooperation for the conversion of a particular product (milk, meat) to edible food (e.g. yoghurt, fermented sausages, olives) which is an example of bacterial interaction (Nychas et al., 2008). The starter culture (two, three or more species of bacteria) used in fermentation process gives best results in boosting flavour and taste development, colour stability, fat rancidity risk reduction as well as texture improvement of the end product. On the contrary, production of antimicrobial substances such as organic acids, bacteriocins and volatile compounds (e.g., diacetyl), competition for nutrients (e.g. limitation or starvation), oxygen or hydrogen sources (in aerobic or anaerobic ecosystems, respectively) that usually inhibit growth, is considered as competitive or negative responses (i.e. antagonistic) of interactions (Drosinos et al., 1997; Pin et al., 2002). In another study of Coleman et al.

(2003), the growth of *E. coli* was affected by the population density of competing microorganisms in ground beef.

At one point it was assumed bacteria acted independently (O'Donnell et al., 2007). However, it is now also evident that, communication and interaction exist among units or cells of a bacterial community growing in the same habitat (Keller and Surette, 2006). Microbes growing in a common habitat are known to strive for growth and survival that forces them to produce an excessive number of secondary metabolites, enabling them to react to a large array of chemicals around their microenvironment (Keller and Surette, 2006).

Growth of a specific bacterium differs in presence of other microorganisms. For example, a study of Russo et al. (2006) reported, the GR of *B. thermosphacta* was about 1.5 log higher when it co-cultured with pseudomonads and LAB (*Lactobacillus sakei, Leuconostoc mesenteroides*), compared to the growth of pure cultures. In contrast, the number of *B. thermosphacta* was lower when incubated with a mixture of LAB and *Enterobacteriaceae*. The ability of *Pseudomonas spp*. to produce siderophores or utilize glucose at faster rate than *Sh. putrefaciens* (*Enterobacteriaceae)*, enabled the former to dominate over the latter species (Gram and Dalgaard, 2002). Indeed, from the study of Nychas et al. (2007), the significance of microbial interactions in food spoilage in governing the growth of spoilage microflora can be better understood.

As mentioned earlier, the final composition of the microbial community determines the pattern of spoilage, as the spoilage process involves the growth of microorganisms to certain levels (log 7- log 9 CFU/g), which communicate and control the growth of each other (Boddy and Wimpenny, 1992). Such interactive behaviour among microorganisms in food

ecosystems can be distinguished as being counterproductive or favourable, and the interactions are antagonistic or competitive as well as metabiotic or cooperative (Fredrickson, 1977).

2.7.1 Antagonism and competitive

Interference or antagonistic effects of some bacterial strains on the growth of others results in complex interactive networks in natural environments and are thought to influence community structure and also to maintain cohesion of bacterial populations (Cordero et al., 2012; Giudice et al., 2007; Long et al., 2013; Perez-Gutierrez et al., 2013; Prasad et al., 2011; Rypien et al., 2010; Validov et al., 2005; Vetsigian et al., 2011). Food spoilage microorganisms manifest their antagonistic abilities by changing the environmental conditions, e.g. by producing lactic acid and resulting in a change of pH, antibacterial peptides (bacteriocins) or NH_3 and trimethyl-amine (Adams and Nicolaides, 1997; Ellis et al., 2000). By altering the environmental conditions as well as by producing such toxic compounds, a powerful way is established to supress the growth of other bacteria and develop a discriminate dominance (Gram et al., 2002). However, microbial antagonism relies potentially on nutrient sources as these are utilized by increasing bacterial population, which was first demonstrated by Monod (1949), who revealed the connection between depletion of nutrition concentration with the increase of bacterial growth.

However, this interactive network is not constant and varies between sites and taxa, making it a productive study area in bacterial community ecology and thus needs more research focusing in this specific area (Wobeser et al., 2014).

Bacteriocins

Production of bacteriocins is one important and widely studied phenomenon of competitive interaction of LAB, resulting in suppression of growth of pathogenic and spoilage bacteria (Amortegui et al., 2014; Wallace et al., 2011). Initial studies focused on bacteriocin production by LAB associated with dairy products, but later this antagonistic effect was also determined for similar species isolated from meat (De Martinis et al., 2001; Oliveira et al., 2008).

Specific characteristics of inhibition make bacteriocins of great interest as food biopreservatives. Jeevaratnam et al. (2005) highlighted that, a reduction in maximum cell density of *Listeria monocytogenes* was observed when LAB was used as a dominant bacterial flora in lightly preserved foods. This reduction in maximum population density of a specific organism by a dominant one is termed as "Jameson effect" (Jameson, 1962; Ross et al., 2000; Stephens et al., 1997).

Bacteriocins are ribosomally synthesized heat-stable low molecular weight peptides retaining antimicrobial activity against a range of specific bacteria, which are closely related to the producer strain (Amortegui et al., 2014; Cotter et al., 2013). The mechanism of action of these peptide molecules involves physical damage of other bacterial cells by pore formation in the cell membrane of target microorganisms and wreckage of essential biosynthesis of protein and expression of genetic material (Cotter et al., 2013).

Based on structure and stability bacteriocins can be divided into four classes (Klaenhammer, 1993). Class 1 bacteriocins are low molecular weight peptides, also termed as lantibiotics which are typically composed of 19 to 50 amino acids (Parada et al., 2007; Yang et al., 2014). These bacteriocins are further divided into class 1a and class 1b. Nisin A is the most widely

studied isolated from LAB bacteriocins belongs to class 1a and composed of pentacyclic peptide of 34 amino acid residues (Parada et al., 2007). Class 2 are the bacteriocins with small size, heat stable, non-modified peptides are subdivided into 2a, 2b, 2c. Pediocin like Listeria active peptides belong to class 2a and class 2b contains bacteriocins constituting two different peptides. Class 2c are supposed to secret by the general sec- system (Nes et al., 1996). Class 3 bacteriocins are large and heat labile whereas Class 4 forms large complexes with other macromolecules (proteins, lipids and carbohydrates) (Klaenhammer, 1993; Yang et al., 2014).

From a study of Nes et al. (1995), it was established that, at least four different genes of general genetic structure encoding basic functions for extracellular antimicrobial activity are involved in the secretion of cationic peptide bacteriocins and those genes are usually organized in operon clusters. It is quite common to produce multiple bacteriocins by a single bacterium. Quadri et al. (1995), for instance, found that, *Carnobacterium piscicola* LB17B produced two different bacteriocins, one of them was encoded by plasmid and the other by chromosome.

2.7.2 Metabiosis (cooperative interaction)

The nutrient contribution of one organism to another, or the generation of suitable conditions to promote a beneficial effect, is termed as mutualism or cooperative interaction between two microbes. For instance, oxygen removal by Gram-negative microflora undoubtedly promotes the growth of anaerobic organisms, e.g. *Clostridium botulinum*, or synthesis of biosurfactants by *Pseudomonas spp.* that enhance growth of other strains of bacteria in chicken meat (Gram et al., 2002; Mellor et al., 2011). Likewise, it is also found from previous studies that initial addition of different Gram-negative psychrotrophic

bacteria in milk eventually results in higher amount of acid from LAB (Gram et al., 2002). Such a mutualistic relationship benefits both microorganisms involved, and this kind of nutrient interdependency could play a key role in spoilage of food (Jorgensen et al., 2000). However, in these interactions, the competency of the microorganisms involved is very important (Pande et al., 2015).

Borch et al. (1996), demonstrated that single cultures of LAB and *Hafnia alvei* do not produce off-odours in beef, whereas co-culture of both bacteria produced typical spoilage off-odours in VP beef. Similarly, putrescine production was intensified 6-15 times more whenever arginine deaminase positive LAB was co-cultured in a mixture with ornithine decarboxylase positive *Enterobacteriaceae* in VP beef (Dainty et al., 1986; Gram et al., 2002). Cooperative interactions are highly regulated by the structural configuration of the microbial community to facilitate the exchange of diffusible nutrients and signal communication (Pande et al., 2015). Bacteria can interact in a number of ways, e.g. constructing outer membrane vesicles or they can be connected via channels, nanotubes or pili for intercellular connections, to interchange and fulfil each other's metabolic requirements (Pande et al., 2015). Cell-to-cell connectivity offers different other potential advantages, such as transfer of genetic materials (plasmids), social networking by chemical signals within communities, and the supply of essential proteins for social communication or defence mechanism (Pande et al., 2015).

2.7.3 Quorum-sensing

Quorum-sensing can be defined as a mechanism by which bacteria communicate information about cell density in their environment, in order to regulate gene expression (Keller and Surette, 2006). It involves the production and accumulation of signalling

molecules that are also known as autoinducers (AI) (Skandamis and Nychas, 2012). This system of production and accumulation of autoinducers affects synchronized performance in cell density (Kleerebezem et al., 1997).

Two factors are important for the occurrence of true communication in quorum-sensing. Firstly, the production of chemical signals by one organism and recognition of those signals by other party and secondly, the adaptation of signal receivers in response to the signal (Keller and Surette, 2006). Since producing a signal has a fitness cost, natural selection stands in opposition to signal production. This depends on whether the adoption of changed behaviour of receiver produces an advantageous effect for the emitter. Simply, cooperation between two bacteria develops and progresses, as well as remains stable, only when both benefit from transferred messages communicated through chemical signals (Keller and Surette, 2006). In addition, the bacterial strains in cooperation rely on 'kin selection', a selective force to promote interspecies interaction (Hamilton, 1964)

Cell-to-cell signalling, or quorum-sensing, involves three types of signal molecules (Keller and Surette, 2006).

In Gram-positive bacteria, oligopeptides serve as a signal molecule to monitor the population size. Usually, a precursor of protein is synthesized, converted into N-active signalling peptide and is then released from the cell (Kleerebezem et al., 1997). The chemical network of the signal is very specific and is determined via the amino acid sequence specific one strain of the same species (Ji et al., 1997; Lyon et al., 2002).
The most widely and intensely studied signal molecules are the N-Acyl Homoserine Lactones (AHLs) and these molecules are very significant in Gram-negative bacteria (Eberl, 1999; Fuqua et al., 1996; Whitehead et al., 2001).The accumulation of AHLs in vacuum-packaged

beef should be considered as the level of AHLs is interrelated to the growth of *Enterobacteriaceae* (Ravn et al., 2001). Undoubtedly, the participation of these organisms is important in the spoilage process (Borch et al., 1996). Moreover, Nychas et al. (2007), reported that AHL compounds were also identified in minced meat and meat fillets at spoilage level produced by some identical spoilage microflora isolated previously from fresh meat. Although the specific role of AHLs in muscle food spoilage remains ambiguous, Gram & Dalgaard (2002) stated that, some phenotypes of food spoilage bacteria such as pectinolytic, lipolytic, proteolytic, chitinolytic characteristics were associated with AHLs regulation.

SAM (S-adenosylmethionine) reacts with an acyl–acyl carrier protein (acyl–ACP) during the synthesis of AHLs. This reaction is catalysed by an enzyme of LuxI family (Eberl, 1999; Fuqua et al., 2001; Greenberg, 2003). The specificity of this mechanism is at moderate level, along with the production of predominant and precise one AHL, two or more minor AHLs may also produce. Previous study showed that, Las I (AHL synthase of *Pseudomonas aeruginosa*) produced both 3-oxohexnoyl-homoserine lactone and 3-oxodoecanol-homoserine lactone (Pearson et al., 1994; Winson et al., 1995). However, the signal is generally distinguished by a number of transcriptional regulators of LuxR family (Shaw et al., 1997).

Both the Gram positive and Gram-negative bacteria posssess LuxS/ AI-2 pathway (Surette et al., 1999). The signal molecule produced by all strains are 4,5-dihydroxy-2,3-pentanedione which is identical and common (Burgess et al., 2002; Schauder et al., 2001).

2.8 Predictive model and quantitative description of bacterial interaction

Factual quantitative science that develops mathematical equations and models by elucidating and interpreting microbial behaviour under variables of environmental

conditions (e.g. physical, chemical, competitive) is regarded as predictive microbiology. This includes predictive model as tool for estimating food spoilage and freshness (Koutsoumanis and Nychas, 2000; Mataragas et al., 2006). The models and equations can also be used to explain the specific role of food technique, maintenance during transportation as well as preservation requirement on microbial growth (McMeekin et al., 1997). Microbial models have a number of applications in Hazard Analysis and Critical Condition Points (HACCP), microbial shelf-life studies, risk assessment, temperature function integration and meat hygiene regulatory activity, product research and development, technical and nontechnical education (Larsen et al., 2012; Pin and Baranyi, 1998). With the incorporation and advent of multifactorial predictive models, the safety and quality of meat can be better assured for the beef industry (Nychas et al., 2008). Within predictive modelling, secondary models deal with the effect of intrinsic (pH, aw, redox potential, nutrient content, antimicrobial properties) and extrinsic (gaseous atmosphere, temperature) on bacterial growth within a food matrix.

Microbial interactions in model development can be incorporated to predict mechanisms and levels of metabolism of two or more taxonomic group of microorganisms interacting with one another within variables of their environment parameters (Gram et al., 2002). While developing such models for microbial communities, dynamic must be considered that include functional and taxonomic dynamics (Larsen et al., 2012). Taxonomic diversity is a degree of the constitution of community species that is conserved or changed through interactions and transformations between individual strains and their environment. Functional diversity is a level of the persistence and variety of predicted enzyme activity that is encoded in a community's metagenome and stands for the capacity to manifest a physical

expression that interacts with a selective environmental condition (Larsen et al., 2012). From a study of O'Donnell et al. (2007), every single microbial cell is assumed to act as an individual unit of the model and interacts with environmental restrictions. However, consideration of independent-based methods to whole microbial association needs comprehensive, precise and realistic information about biochemical metabolism of microorganism and their microenvironment characteristics (Ferrer et al., 2008; Freilich et al., 2011).

Models considering microbial interaction can be used to predict mechanisms and levels of metabolism of two or more taxonomic group of microorganisms interacting with one another within variables of their environment parameters (Gram et al., 2002). Dynamic changes in microbial composition and function still need to be considered for model development (Larsen et al., 2012).

Microbial composition includes the taxonomy and diversity and whether over time these are conserved or changed due to interactions between individual strains and the changing meat environment. Functional diversity relates to the persistence and variety of predicted enzyme activity that is encoded in a community's metagenome. Understanding this also requires knowing how rapidly enzymes act in relation to the environmental condition (Larsen et al., 2012).

2.9 qPCR technique to quantify bacterial load

Advancements in molecular methods, specifically qPCR technique, has been identified as a subject of excellent alternate of conventional culture-based method for detection, quantification and study microbial diversity in food environment (Le Drean et al., 2010;

Postollec et al., 2011). Several advantages offered by this method including high sensitivity, specificity, simultaneous detection of different organism in the same reaction enabled qPCR as a powerful, convenient and reliable tool (Espy et al., 2006; Postollec et al., 2011). The presence of low concentrations of spoilage organism during processing step which proliferate during storage impacting negatively on beef quality and safety is inevitable (Bell and Garout, 1994; Borch et al., 1996). Such effects can be eliminated by adoption of a rapid identification method for early detection and initial quantification of spoilage organism. Detection chemistry of qPCR is quite similar to conventional PCR except from the requirement of post amplification manipulation (Kralik and Ricchi, 2017). Reaction progression can be monitored in real time by detection fluorescent labelled target DNA amplification (Kralik and Ricchi, 2017). At a certain cycle number fluorescence of an amplifying PCR product will exceed a specified background level. This cycle number is thus known as the cycle threshold (C_t). C_t value is thus directly associated with the quantity of newly synthesized product in qPCR reaction (Hanna et al., 2005; Mackay, 2004). Determination and quantification of target DNA sequence determines the total microbial load in a sample easily (Ibekwe et al., 2002). Moreover, simultaneous detection of more than a single isolate in a single reaction by analysing melt curves and using numerous fluorophores is feasible with the qPCR method (Hanna et al., 2005; Omiccioli et al., 2009). A number of qPCR methods have already been developed for the detection of spoilage organisms from food matrices, however, routine practice of identification and quantification of spoilage organisms at initial stage of processing would be beneficial for meat industries to identify and quantify pathogenic and spoilage organism of beef and beef products.

2.10 Conclusions

Globally, a remarkable amount (3.5 billion kg) of meat and meat products are spoiled every year during processing and distribution steps, which implies considerable economic and environmental impact (Dave and Ghaly, 2011). Storage under chilled condition and VP are two main strategies to extend the shelf-life of long-term preserved foods, such as beef. The potential of this packaging method lies in preventing the growth of spoilage bacteria that are frequently present on meat. However, further research is required toward the development of microbial community and metabolism to understand meat spoilage.

It has been proven that bacteria make use of intercellular connections (communication) to exchange nutrients and enhance each other's competency. This potential theory can be effective for the determination of advanced preservation methods of fresh meat by performing a practical view of interactions among spoilage bacteria, toward the progression of spoilage in situ on VP beef. Moreover, communication involves a cost in terms of energy while producing signals, and the level of interaction varies on different intrinsic, extrinsic factors as well. Thus, it is crucial to investigate the parameters at which interaction among three or more bacteria e can influence others to accelerate or inhibit growth individual expense.

Despite limitations, predictive models can provide authentic and economic estimation for expeditious shelf-life determinations. For this purpose, two types of models need to be incorporated, primary models to assess the GR of SSOs over time, and secondary models to demonstrate the environmental effects on growth kinetic parameters.

Significantly, validation of developed models under dynamic storage temperature conditions can be an extremely functional source of information for the shelf-life predictions in the area of quality regulation network of meat industries. However, the role of environmental factors to regulate bacterial interactions inside VP beef matrix and consideration of these interactive behaviour while developing predictive model to assess bacterial growth are yet to be determined and need more research attention. Development of such accurate predictive models will be useful to categorise the behaviour and kinetics of major group of spoilage microorganism, which can eventually be used as industry tools to estimate shelf-life of various foods.

Chapter 3. Effect of glucose, pH and lactic acid on *Carnobacterium maltaromaticum, Brochothrix thermosphacta* and *Serratia liquefaciens* within a commercial heat-shrunk vacuum-package film.

3.1 Abstract

Carnobacterium maltaromaticum, Brochothrix thermosphacta and *Serratia liquefaciens* are common spoilage organisms found within the microbiome of refrigerated vacuum-packaged (VP) beef. Extending and predicting VP beef shelf-life requires knowledge about how spoilage bacteria growth is influenced by environmental extrinsic and intrinsic factors. Multifactorial effects of pH, lactic acid (LA) and glucose on growth kinetics were quantified for *C. maltaromaticum, B. thermosphacta* and *S. liquefaciens* within a heat shrink-wrapped VP commercial film containing a simulated beef medium. LA, pH, and undissociated lactic acid (UDLA) significantly affected bacterial growth rate (p <0.001), whereas 5.55 mM glucose produced a marginal effect. At 1.12 mM UDLA, growth rate and maximum population density decreased 20.9 and 3.5%, 56 and 7%, and 11 and 2% for *C. maltaromaticum*, *B. thermosphacta,* and *S. liquefaciens*, respectively.

3.2 Introduction

Fresh meat is a highly perishable food that supports microbial growth due to favorable pH, water activity and protein (Ercolini et al., 2006). Meat spoilage is an ecological phenomenon (Nychas et al., 2008), signified by off-odours, off-flavours, slime, discoloration, and undesirable texture attributed to microbial, chemical and enzymatic effects (Gram et al., 2002; Jackson et al., 1992).

Although much has been reported about environmental effects on spoilage bacterial growth for aerobic and vacuum-packaged (VP) meat (Borch et al., 1996; Ercolini et al., 2009; Iulietto et al., 2015; Lambert et al., 1991; Nychas et al., 2008; Stellato et al., 2016), few if any studies have quantified growth kinetics of individual species within commercial heat shrink-wrapped VP films, used by meat processors to improve shelf-life, appearance, handling, leak rate, and drip loss (Bell et al., 2001). Consequently, we lack important information about the impact of low oxygen environments (5 cc O_2/m^2/day) on bacterial growth compared to the numerous reports that used non-heat-shrunk films (20 cc O_2/m^2/day).

For VP beef, water activity is relatively stable (Seidman et al., 1976), in contrast to pH, glucose and lactic acid (LA) that change over time due to microbial and muscle tissue metabolism (Dave and Ghaly, 2011; Garcia-Lopez et al., 1998; Lambert eta al., 1991). Over VP beef shelf-life, pH generally ranges from 5.5-5.8, LA from 0.9-1%, and glucose from 0.1-0.15% (Dainty et al., 1979; Garcia-Lopez et al., 1998; Gill, 1986; Gill and Newton, 1978, 1982; Lambert et al., 1991; Nychas et al., 1998; Nychas et al., 1988). Such information can inform experimental designs to test multifactorial effects of pH, LA and glucose on spoilage bacteria growth kinetics.

Atmospheric conditions change within a refrigerated commercial VP film, shifting from high to low oxygen from air evacuation, sealing, and film gas transfer (Cutter, 2002; Lambert et al., 1991). This typically transitions an initial dominant *Pseudomonas* spp. microflora to one of lactic acid bacteria (LAB) (Narasimha Rao and Sachindra, 2002; Seidman and Durland, 1983), such as *Carnobacterium* spp., a genus commonly associated with stable sensory qualities and extended shelf-life of VP meats (Casaburi et al., 2011; Casaburi et al., 2015; Youssef et al., 2014a; Youssef et al., 2014b). For example, Kaur et al., (2017b), using 16S

rRNA gene analysis of VP beef stored at -0.5˚C for 26 weeks, observed that *Carnobacterium* spp. were dominant species at later stages of storage. Also, *Carnobacterium* spp*., Serratia* spp. and *Hafnia* spp. predominated in VP lamb stored at -1.2 and 8˚C, with the former two species reaching 10^6-10^8 CFU/cm^2, compared to only 10^4-10^5 CFU/cm^2 for *Brochothrix* and *Pseudomonas* spp. (Kaur et al., 2017a).

Carnobacterium spp. can have beneficial effects on shelf-life and safety, such as by suppressing growth of pathogenic and spoilage bacteria through actions of un-disassociated lactic acid (UDLA) and bacteriocins (Afzal et al., 2013; Brillet et al., 2005; Duffes et al., 1999; Leisner et al., 2007; Martin-Visscher et al., 2008). Also, *C. maltaromaticum* produces a desirable malty, chocolate-like aroma through catabolism of leucine, enhancing flavor (Afzal et al., 2012). In contrast, LAB sometimes cause food spoilage via excessive production of LA, CO_2, slime and metabolites (e.g. acetoin, tyramine, butanoic acid and 1-octen-3-ol) (Amezquita and Brashears, 2002; Borch et al., 1996; Casaburi et al., 2011; Hoyle et al., 2009; Laursen et al., 2005; Leisner et al., 1995; Smith et al., 2005).

Undesirable species of refrigerated VP meat communities include *Brochothrix thermosphacta*, which is associated with 'sweaty socks/sour/sweet/cheesy' odor from glucose fermentation (Casaburi et al., 2015), as well as cold-tolerant *Enterobacteriaceae*, in particular *Serratia liquefaciens,* which produces 'foul-smelling' odors from diamines and sulfuric compounds (Chaves et al., 2012; Dainty et al., 1986; Edwards et al., 1985; Gill and Penny, 1988).

Although proper refrigeration and packaging are key quality controls for beef processors, few if any additional science-based interventions are used to produce desirable microbial

communities. However, this problem could be remedied, in part, by facilitating formation of beneficial microbial communities. To achieve this goal, a quantitative understanding of how specific environmental factors influence the behavior of desirable and undesirable spoilage bacteria is required.

Although reports describe growth kinetics of spoilage bacteria in VP beef (Casaburi et al., 2011; Dainty et al., 1979; Ercolini et al., 2011; Small et al., 2012; Zhang et al., 2015), the majority have examined whole endogenous microbiomes. Consequently, it is not possible to separate the effects of specific environmental factors on species interactions without first understanding bacterial behavior in a single culture system. To address this knowledge gap, we quantified the effects of pH, glucose, and LA separately for *C. maltaromaticum, B. thermosphacta* and *S. liquefaciens*, using a simulated beef matrix within a heat shrink-wrapped commercial VP film.

3.3 Materials and methods

3.3.1 Bacterial strains

C. maltaromaticum (C0a), *B. thermosphacta* (A8f) and *S. liquefaciens* (D0d) were previously isolated from VP beef primals stored at -0.5˚C for up to 30 weeks, obtained from different abattoirs in Australia (Small et al., 2012). Isolates were transferred from a -80˚C freezer, streaked on tryptone soya agar (TSA, Oxoid Ltd., Thebarton, Australia) and incubated at 25˚C for 48 h. [Appendix A describes the procedures and criteria used to select the three specific isolates.]

3.3.2 Inoculum preparation

Single colonies from TSA were inoculated into brain heart infusion broth (BHI, Amyl Media Ltd., Melbourne, Australia) and incubated at 25˚C for 24 h. The OD_{600} was spectrophotometrically measured (SPECTRO star Nano, Victoria, Australia) and adjusted to a value of 0.1 with sterile BHI broth, representing approximately 10^8 CFU/ml. By serial dilution, bacterial cell concentration was adjusted to approximately 10^5 CFU/ml.

3.3.3 Media formulation

Levels for pH, lactic acid, and glucose were based on reports for post-rigor beef muscle (Farber and Idziak, 1982; Garcia-Lopez et al., 1998; Gill, 1986). Specifically, these were: pH (5.5, 6.5), LA (0 mM, 50 mM) and glucose (0 mM, 5.55 mM), used in eight formulations of modified BHI broth (mBHI), in a full factorial design (2 × 2 ×2) for two separate trials (Table 3.1). mBHI was prepared from a basal medium of BHI broth without glucose (AM 11-NG, Amyl Media, Ltd., Melbourne, Australia), which contained 10 g blended peptone No. 1, 5 g `sodium chloride, 17.5 g brain heart infusion solid and 2.5 g disodium hydrogen orthophosphate, per liter. L (+) LA (Scharlab, Barcelona, Spain) and D (+) glucose (Sigma, St. Louis, MO, USA) were used to prepare eight formulations of mBHI for each trial. pH was adjusted to 5.5 and 6.5 (+/- 0.05) using 10 M NaOH or 10 M HCl before and after the formulated media were autoclaved. A randomized order of test media was used. Undissociated LA (UDLA) was calculated by the formula: $[UDLA] = [LA]/ (1+ 10^{(pH-3.86)})$.

Table 3.1 Modified brain heart infusion (mBHI) broth formulations

Medium	Trial	LA (mM)	Glucose (mM)	pH	UDLA (mM)
1	1,2	0	0	5.5	0
2	1,2	0	0	6.5	0
3	1,2	0	5.55	5.5	0
4	1,2	0	5.55	6.5	0
5	1,2	50	0	5.5	1.12
6	1,2	50	0	6.5	0.11
7	1,2	50	5.55	5.5	1.12
8	1,2	50	5.55	6.5	0.11

3.3.4 Preparation of media in commercial vacuum-sealed shrink bags

Fifty milliliters of autoclaved mBHI were aseptically transferred into commercial vacuum barrier shrink bags (Newteq, Cryovac Inc., Sealed Air Corporation, Collinsons Vacuum Packaging, Victoria, Australia) using a sterile measuring cylinder in a laminar flow work station (CLYDE-APAC, model no. HWS-120, NSW, Australia). The bags were typical of those used by Australian meat companies to package beef primals, with an oxygen transmission rate of 20 cc/m^2/day at 23°C, 0% RH and moisture vapor transmission rate of 12 g/m^2/day at 38°C, 90% RH (Cryovac product information sheet). Bags were sealed using a VP machine (Tecnovac, model no. T60 GAS, Grassobbio, Italy), mimicking a commercial protocol of 0% gas, 99% vacuum, and a sealing time of 2 sec. After sealing, bags of media were heat-shrunk in a hot water bath at 84°C for 3 sec, which reduced oxygen transmission rate to 5 cc/m^2/day (personal communication, Cryovac). Potential contamination during the filling and sealing processes was evaluated by incubating bags overnight at 25°C. Thereafter, the outer surface of each bag was wiped with 70% ethanol and a small piece of latex sealing

tape (Stylus Tapes International, Victoria, Australia) adhered to the outside of each bag, and subsequently used as a septum. 100 µL of broth was removed by a sterile syringe through the septum and plated on TSA to confirm broth sterility.

3.3.5 Sample inoculation

Prior to inoculation, the outer surface of the septum was wiped with 70% ethanol and then 500 µL of inoculum injected into two bags per bacterial isolate. Inoculum was mixed by manual massaging bags; 100 µL was immediately aspirated by sterile syringe, diluted and plated to determine initial bacterial concentration. All bags were incubated at 10˚C to simulate moderate temperature-abuse in the commercial supply chain.

3.3.6 Bacterial enumeration

Bags were sampled at 9 to 15 h intervals, depending on bacterial species and media formulation, over a period of 100 to 300 h. At each sampling time interval, septa were wiped with 70% ethanol, and 100 µL broth removed and plated on BHI agar. Another 100 µL was transferred to a micro-centrifuge tube containing 900 µL BHI broth, serially diluted in 10-fold increments, and then plated on BHI agar. BHI agar was incubated at 25˚C for 48 h for *B. thermosphacta* and *C. maltaromaticum*, and for 24 h at 25˚C for *S. liquefaciens*. Colonies were counted and recorded as $\log_{10}$ CFU/ml. A total of 48 growth curves were generated for the three isolates in eight different media with two biological replicates.

3.3.7 Data analysis

DMFit (v3.5 [ComBase; http://www.combase.cc/tools/]) software was used to fit the Baranyi and Roberts (1994) growth model to kinetic data and to measure lag phase duration (LPD), growth rate (GR) and maximum population density (MPD). Kinetic parameters were

subsequently analyzed by the General Linear Model (GLM) procedure in SAS (v 9.4; SAS, Inc., Rockville, MD, USA). A p- value <0.05 was considered significant.

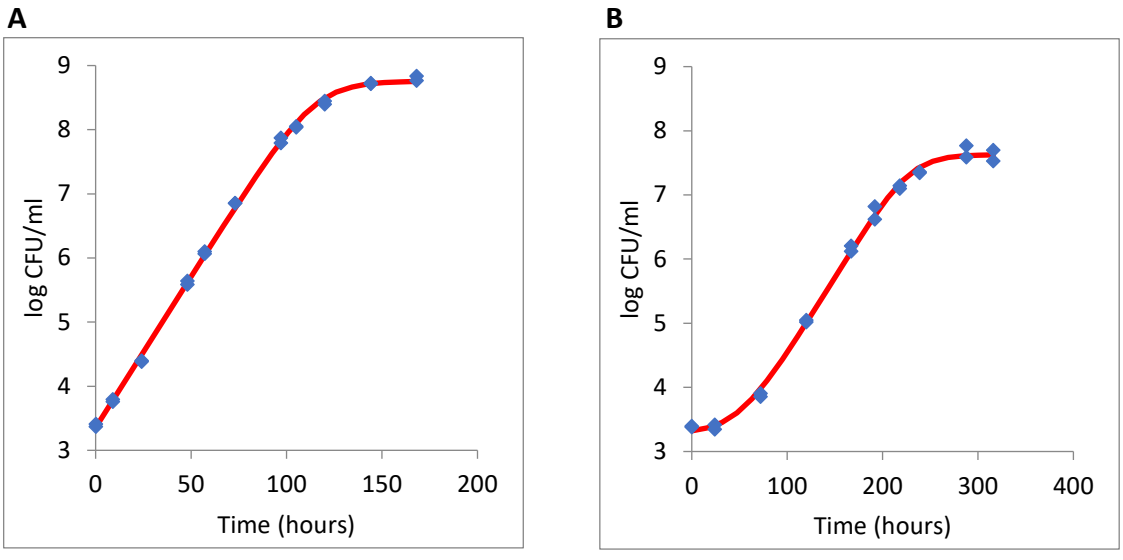
3.3.8 Data deposit

Data are deposited in ComBase and can be accessed for the author name, 'Mohsina'

3.4 Results

Figure 3.1 depicts representative bacterial growth kinetics across different treatments. Among treatments and trials, LPD showed high variation (not shown) and no significant correlations with treatment. Therefore, only GR and MPD were further evaluated. Additional figures of GR and MPD are provided in Appendix A. In general, pH and UDLA produced the most significant effects on GR and MPD, as described in detail below.

In Table 3.2, the average GR and MPD are shown when a single treatment was held constant (i.e. normalized) for each species, while other treatments varied (e.g. glucose, LA, UDLA). For example, the first line of data in Table 3.2 shows the average GR and MPD for all treatments in which the pH was 5.5.



C

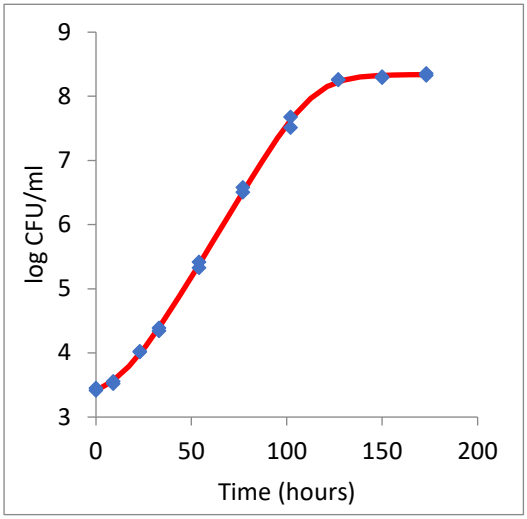


Figure 3.1. Growth of *C. maltaromaticum* (A), *B. thermosphacta* (B), and *S. liquefaciens* (C) in 50 mM LA, pH 5.5, 1.12 UDLA (medium 5) stored at 10°C. Blue markers indicate observed bacterial concentration (log CFU/ml), along with fitted Baranyi and Roberts model.

3.4.1 Carnobacterium maltaromaticum

Reducing pH from 6.5 to 5.5 decreased GR by 40% and MPD by 3.4% (Table 3.2). 50 mM LA reduced GR 9.4% (Table 3.2) and MPD 2.2% (Table 3.2). Glucose (5.55 mM) had no significant effect ($p > 0.05$) on GR or MPD. 50 mM LA at pH 6.5 and pH 5.5 produced UDLA of 0.11 and 1.12 mM, respectively. Growth rate decreased 3.6% at 0.11 mM UDLA, and 20.9% at 1.12 mM UDLA (Table 3.2). 0.11 mM UDLA produced no significant effect on MPD, but 1.12 mM UDLA did, reducing MPD 3.5% (Table 3.2). The interaction of pH and LA had a significant effect on GR and MPD at pH 5.5 but not at pH 6.5.

3.4.2 Brochothrix thermosphacta

pH 5.5 significantly decreased *B. thermosphacta* GR more than 50% and MPD 10% (Table 3.2). 50 mM LA decreased GR 34% and MPD 4% (Table 3.2). 5.55 mM glucose had no

significant effect on GR but had a significant effect ($p<0.05$) on MPD. However, the effect was small, and increased MPD only 1% (i.e. 0.1 log CFU/ml). 0.11mM UDLA (pH 6.5, 50 mM LA) reduced GR 20%, while 1.12 mM UDLA reduced GR 56% (Table 3.2). 0.11 mM UDLA slightly reduced MPD, whereas 1.12 mM UDLA reduced MPD 7% (Table 3.2). pH and LA interactions were significant at both pH levels, for GR and MPD.

3.4.3 Serratia liquefaciens

pH 5.5 significantly decreased GR 39% (Table 3.2) and produced a 3.5% decrease in MPD (Table 3.2), again demonstrating pH as a dominant factor affecting growth ($p <0.001$). A smaller reduction in GR (4%) and MPD (1.5%) was observed with 50 mM LA (Table 3.2). In contrast, glucose did not affect GR or MPD ($p> 0.05$). UDLA reduced GR 1% at 0.11mM, and 11% at 1.12 mM UDLA (Table 3.2). A moderate 2% decrease in MPD occurred with 1.12mM UDLA, with no significant effect when 0.11mM was present (Table 3.2). There were no interactive effects between pH and LA on GR or MPD at pH 6.5, whereas pH 5.5 exhibited effects at both levels of LA, affecting GR and MPD significantly.

Table 3.2 Effects pf pH, LA, glucose and UDLA on GR and MPD of *C. maltaromaticum*, *B. thermosphacta*, and *S. liquefaciens*.

Species	Condition	Level	GR (log CFU/h)	MPD (log CFU/ml)
Carnobacterium maltaromaticum	pH	5.5	0.053 [b*]	8.695 [b]
	pH	6.5	0.088 [a]	9.005 [a]
	LA	0 mM	0.074 [a]	8.945 [a]
	LA	50 mM	0.067 [b]	8.755 [b]
	pH, LA	6.5, 0 mM	0.089 [b]	9.036 [a]
	pH, LA	6.5, 50 mM (0.11 mM UDLA)	0.086 [a]	8.974 [a]
	pH, LA	5.5, 0 mM	0.058 [b]	8.855 [a]
	pH, LA	5.5, 50 mM (1.12 mM UDLA)	0.046 [c]	8.536 [b]
Brochothrix thermosphacta	pH	5.5	0.037 [b]	7.909 [b]
	pH	6.5	0.081 [a]	8.804 [a]
	LA	0 mM	0.071 [a]	8.518 [a]
	LA	50 mM	0.047 [b]	8.195 [b]
	pH, LA	6.5, 0 mM	0.090 [a]	8.854 [b]
	pH, LA	6.5, 50 mM (0.11 mM UDLA)	0.072 [a]	8.754 [a]
	pH, LA	5.5, 0 mM	0.052 [a]	8.182 [b]
	pH, LA	5.5, 50 mM (1.12 mM UDLA)	0.023 [b]	7.637 [c]
Serratia liquefaciens	pH	5.5	0.055 [b]	8.561 [b]
	pH	6.5	0.090 [a]	8.875 [a]
	LA	0 mM	0.074 [a]	8.785 [a]
	LA	50 mM	0.071 [b]	8.650 [b]
	pH, LA	6.5, 0 mM	0.091 [b]	8.916 [a]
	pH, LA	6.5, 50 mM (0.11 mM UDLA)	0.090 [a]	8.834 [a]
	pH, LA	5.5, 0 mM	0.058 [b]	8.655 [a]
	pH, LA	5.5, 50 mM (1.12 mM UDLA)	0.051 [c]	8.467 [b]

* letters indicate significance within each species

3.5 Discussion

A variety of eco-physicochemical factors impose selective pressure on microbial colonization of meat following initial contamination of the beef carcass, including atmosphere, pH, glucose and LA (Nychas et al., 2008; Pennachia et al., 2011). Although there are extensive publications on beef microbiology, little is known about how low-oxygen environments within commercial heat-shrunk films influence growth of spoilage species.

A broth system was used as the test matrix, due its well-defined composition and greater control over test variables. In contrast, individual pieces of meat would introduce significant error due to spatial variation of physiochemical properties.

3.5.1 pH

Tolerance to lower pH (and lower oxygen) allows *C. maltaromaticum* to progressively dominate the microbiome of VP meat (Egan, 1983). Reasons may include a direct effect of pH on metabolism (growth rate), increased production of inhibitory compounds at pH 5.5 (Zhang et al., 2017), as well as pH-mediated bacterial 'stress' (De Vuyst et al., 1996). Yang et al. (2009) reported *C. maltaromaticum* grew from pH 5.5 to pH 8.2, with maximum GR from 6.2 to 7.5. Also, Zhang et al. (2018) showed that the GR of *C. maltaromaticum* at pH 5.4- and 0-mM LA was 18% lower than at pH 6.2- and 0-mM LA.

pH was the most important factor affecting *C. maltaromaticum*, *B. thermosphacta,* and *S. liquefaciens* GR and MPD, with a more pronounced effect on *B. thermosphacta*. Specifically, at pH 5.5 versus 6.5, there was a two-fold reduction in *B. thermosphacta* GR and a 1-log reduction in MPD. This agrees with Campbell et al. (1979), showing *B. thermosphacta* growth inhibition at pH 5.8 in beef under anaerobic conditions, with a report by Grau (1980)

and another by Papon and Talon (1988) where pH reduction from 7.0 to 5.6 caused a 70% reduction in *B. thermosphacta* growth at 24°C. Leroi et al. (2012) and the ComBase model for *B. thermosphacta* (Baranyi and Tamplin, 2004) describe an optimum pH of approximately 6.8.

S. liquefaciens, the most frequently detected Enterobacteriaceae on fresh and VP meat (Grau, 1981). In our study, *S. liquefaciens* GR at pH 5.5 was approximately 40% of that observed at pH 6.5, similar to a report by Gill and Newton (1979), showing a 30% reduction in *S. liquefaciens* GR at pH 5.7 versus pH 6.5 in VP meat.

3.5.2 Lactic acid

In post-mortem mammalian muscle, LA ranges from 0.9 % (90mM) to 1%, (100mM) (Garcia-Lopez et al., 1998), producing different effects among bacterial species. For example, *C. maltaromaticum* weakly tolerates LA, in contrast to *Leuconostoc mesenteroides* and *Lactococcus lactis* at <10°C (Yang et al., 2009). In addition, a shift in LA from 13,700 µg/ml (150 mM) to 17,100 µg/ml (190 mM), caused a corresponding shift in dominance from *C. divergens* to *L. mesenteroides* in VP beef stored for 16 weeks at -1.5°C (Jones, 2004). For 50 mM LA, we demonstrated a decrease in *C. maltaromaticum* GR and MPD. Other studies report a 1-log reduction in LAB growth (MPD) in VP beef treated with 2% (200mM) LA (Signorini et al., 2006). Zhang et al. (2018) reported that *C. maltaromaticum* GR at pH 5.4 was 54% lower at 60 mM LA compared to 0 mM LA.

B. thermosphacta was most sensitive to LA, among the test species, showing a 0.5 log CFU/h and 0.31 log CFU/ml reduction in GR and MPD, respectively, similar to findings of Grau (1980).

S. liquefaciens was least sensitive to LA, i.e. 50 mM moderately reduced GR and MPD by 0.004 CFU/h and 0.13 log CFU/ml, respectively. Gill and Newton (1982) found that, along with other Gram-negative bacteria, only three of 59 *S. liquefaciens* strains were inhibited in media containing LA at pH 5.5 and 2°C. Grau (1981) also found that L-lactate (100-150 mM) significant controlled growth of fermentative Gram-negative bacteria, including *S. liquefaciens*.

3.5.3 Glucose

Glucose in post-mortem meat tissue ranges from 0.1–0.15% (5.5-8.3 mM) (Gill, 1986), which spoilage organisms can ferment to LA. As a result, tissue pH is reduced, and microbial growth may be restricted (Haavik, 1974; Solé et al., 1994). Nychas et al. (1988) found that under anaerobic conditions, bacterial species preference for glucose catabolism occurs in the order: Enterobacteriaceae > *B. thermosphacta* > *Lactobacillus* spp.

In the present study, 5.55 mM glucose did not significantly increase GR of *B. thermosphacta*, *S. liquefaciens* or *C. maltaromaticum*, but did increase *B. thermosphacta* MPD. Higher levels of glucose (e.g. 55 mM), however, increase *B. thermosphacta* GR (Papon and Talon, 1988) but not that of *S. liquefaciens* (11.1 mM) (Collins-Thompson et al., 1982).

3.5.4 UDLA

Two mechanisms responsible for bactericidal activity of organic acids (Janssen et al., 2007) include: 1) reducing extracellular pH when dissociated protons are released, and 2) when undissociated LA diffuses into bacterial cells and alters cell metabolism (Van Maris et al., 2004). The degree of proton dissociation depends on pH of the environmental matrix (Gill, 1986). In the present study, 1.12 mM UDLA (pH 5.5- and 50-mM LA) markedly inhibited *C.*

maltaromaticum GR and MPD. Similarly, 1.12 mM UDLA reduced GR (approximately 3-fold) and MPD (>1 log CFU/ml) of *B. thermosphacta*. Grau (1980) also reported that pH ≤ 5.7 resulted in higher levels of UDLA that inhibited anaerobic growth of *B. thermosphacta*.

Organic acids above pH 6.0 generally do not inhibit spoilage of meat by Gram-negative psychrotrophs (Gill and Newton, 1982), and *S. liquefaciens* does not grow anaerobically at pH 5.5 with 100-150 mM lactate (Grau, 1981). Likewise, we found 1.12 mM UDLA (pH 5.5- and 50-mM LA) inhibited *S. liquefaciens* GR and MPD (0.5 log CFU/h and 0.37 log CFU/ml, respectively) under VP conditions.

Initial contaminating microorganisms of meat utilize glucose 6-phosphate and glucose as carbon and energy sources (Gill and Newton, 1978). Microbial metabolism of glucose produces LA via the glycolysis pathway, which ultimately reduces meat pH and produces higher concentrations of UDLA (Gill and Newton, 1982). In agreement, we found that for all three test species, the addition of LA significantly reduced growth rate and MPD at pH=5.5. However, at the less stringent conditions of pH=6.5, the reduction was significant only *for B. thermosphacta* (Table 3.2).

These studies expand our knowledge of bacterial growth in low oxygen environments produced by commercial heat-shrunk films (5 cc O_2/m^2/day), compared to reports of non-heat-shrunk VP films (20 cc O_2/m^2/day) that dominate the literature. As a result, we demonstrate that *S. liquefaciens* has the greatest potential for growth within heat-shrunk films, with *B. thermosphacta* being most sensitive. Additional studies are needed to describe spoilage bacteria behaviour in mixed culture systems, which can inform development of novel interventions that increase beef shelf-life.

Chapter 4: qPCR quantification of *Carnobacterium maltaromaticum*, *Brochothrix thermosphacta*, and *Serratia liquefaciens* growth kinetics in mixed culture.

4.1 Abstract

Quantifying growth kinetics of specific spoilage microorganisms in mixed culture is required to describe the evolution of food microbiomes. A qPCR method was developed to selectively amplify individual meat spoilage bacteria, *Carnobacterium maltaromaticum, Brochothrix thermosphacta* and *Serratia liquefaciens*, within a broth medium designed to simulate the composition of beef. An optimized method of DNA extraction was produced for standard curve construction. Method specificity was determined by individual single peaks in melt curves. Reaction efficiency for standard curves of *C. maltaromaticum, B. thermosphacta* and *S. liquefaciens* was high (R^2 = 0.98-0.99), and linear quantification was achieved over a 5 log CFU/ml range. Coefficient of variation was calculated considering both threshold cycle (C_t) and bacterial concentration; the value did not exceed 14% for inter- or intra-runs for either method. Comparison of growth kinetic parameters derived from plate count and qPCR showed no significant variation (P>0.05) for growth rate (GR) and maximum population density (MPD); lag phase duration (LPD) was not included in this comparison due to high innate variability. Log quantification of each isolate was validated in a mixed-culture experiment for all three species with qPCR and plate count differing less than 0.3 log CFU/ml (average 0.10 log CFU/ml, R^2 = 0.98).

4.2 Introduction

Culture-based enumeration methods (e.g. agar-plating) are the “gold standard” when measuring microbial numbers in bacteriological media and food samples (Liu et al., 2019; Lungu et al., 2012; Wiemer et al., 2011), and are relatively simple when enumerating a single species or strain. However, these same methods often fail when applied to mixed culture systems of two or more species, particularly when selective or differential media are not available. Thus, more direct and discriminating enumeration methods are needed to study the dynamics of bacterial spoilage communities.

Culture-based microbiological methods are labour intensive, tedious, and require relatively long processing times (e.g. 4-8 days) (Juneja et al., 2007; Kawasaki et al., 2005; Khan et al., 2014; Uyttendaele et al., 2003). Other potential drawbacks include lack of sensitivity and efficiency to simultaneously detect specific organisms, particularly in the presence of high concentrations of background microorganisms (Liu et al., 2019; Noviyanti et al., 2018; Wiemer et al., 2011). Moreover, stressed bacteria may not readily grow on or in selective media (Hoadley and Cheng, 1974; Law et al., 2015; Noviyanti et al., 2018; Özkanca et al., 2009). Along with these complications, there may be significant variation in growth rates (e.g. 10-20% slower) between wild type and induced antibiotic resistant strains, compromising interpretation of growth kinetic studies (Huang, 2010).

In contrast, molecular methods are rapid, sensitive, and accurate (Ceuppens et al., 2014; Postollec et al., 2011). Frequently used techniques include quantitative real time polymerase chain reaction (real time PCR or qPCR), DNA microarrays, loop-mediated isothermal amplification (LAMP), and DNA hybridization (Zeng et al., 2016). qPCR is an

excellent alternative to culture-based methods due to ease-of-use, and high specificity and sensitivity (Liu et al., 2019).

Using qPCR, an exponential increase in PCR product (amplicon) is monitored in real-time by observing fluorescence intensity at the end of each cycle (Heid et al., 1996; Higuchi et al., 1992). However, the reliability and accuracy of qPCR requires careful optimization and thorough testing of each step (e.g. sample preparation, amplification and data expression), since minor variations may produce erroneous results.

qPCR has been used to measure the growth kinetics of food borne pathogens, such as *Salmonella enteritidis* in chicken juice (Noviyanti et al., 2018), *Listeria monocytogenes* in vacuum-packaged pork (Ye et al., 2013), *Staphylococcus aureus* in pork (Guan et al., 2017), and *Salmonella* spp. in pasteurized and non-pasteurized milk (Kawasaki et al., 2014). Most of these studies applied qPCR to the growth of individual organisms amongst background microbiota, a difficult if not non-feasible approach when applying culture-based methods.

qPCR is advantageous for understanding how environmental factors influence inter- and intra-species interactions during evolution of spoilage communities. To our knowledge, no qPCR method has been reported that can quantify growth of spoilage bacteria in mixed culture. Towards this goal, a dynamic range of quantification (7-8 $\log_{10}$) (Bustin et al., 2005) and multiplex amplification of several target organisms in a single reaction has been reported (García-Cañas et al., 2004).

Using a mixed-culture beef simulation broth system, this study reports a species-specific qPCR method that quantifies three individual species (*Carnobacterium maltaromaticum*, *Brochothrix thermosphacta* and *Serratia liquefaciens*) associated with vacuum-packaged

beef spoilage (Borch et al., 1996; Casaburi et al., 2011; Casaburi et al., 2015; Nychas et al., 2008).

4.3 Materials and Methods

4.3.1 Preparation of bacterial strains for 16S rRNA gene sequencing

Three bacterial strains *C. maltaromaticum* (C0a), *B. thermosphacta* (A8f), *S. liquefaciens* (D0d), previously isolated from vacuum-packaged beef primals (Small et al., 2012) produced by Australian abattoirs, were used in this study. Isolates were transferred from a -80°C freezer, streaked on tryptone soya agar (TSA, Oxoid Ltd., Thebarton, Australia) and incubated at 25°C for 48 h.

To verify isolate identity, 1.8 ml of fresh brain heart infusion broth was inoculated with one or two colonies from TSA and incubated for 16 h at 25^{o}C. After incubation, DNA was extracted using DNeasy UltraClean Microbial Kit following the manufacturer's standard protocol (QIAGEN, Chadstone, Australia). Extracted DNA was quantified using a Nano drop spectrophotometer (ThermoFisher Scientific, Wilmington) and then amplified with 16S rRNA gene universal primers 27F (5´- AGAGTTTGATCMTGGCTCAG-3´) and 519R (GWATTACCGCGGCKGCT-3´), with a BIO-RAD T-100 thermal cycler (Hercules CA, USA). The cycle profile was: 10 min at 94^{o}C at start, followed by 30 cycles of 1 min at 94^{o}C, 1 min at 55^{o}C, 1 min at 72^{o}C, and final extension for 10 min at 72^{o}C. The size of the amplicon was evaluated by agarose gel electrophoresis. PCR products were purified using the UltraClean PCR clean-up kit following the manufacturers' protocol (QIAGEN, Chadstone, Australia), and were then sent to Macrogen (Seoul, Korea) for sequencing. BioEdit version 7.0.5.3 software

was used to analyse raw data. The BLASTN function of NCBI compared sequences with their closest match to confirm isolate identity.

4.3.2 Primer design and selection of primer set

Primer 3 plus (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi) software was used to design primers targeting 16S rRNA gene. Five sets of primers for each isolate were generated and synthesized by IDT (Integrated DNA Technology, NSW, Australia). A temperature gradient PCR (55-72^{o}C) was performed to select a specific primer set (Table 4.1) by identifying the highest annealing temperature for a corresponding isolate. The selected primer set for each isolate was cross-checked by PCR with DNA from the other two isolates at the selected annealing temperature, for a reduced cycle number.

Table 4.1. Primer sets for selective amplification of three bacterial isolate

Bacterial species	**Primer (5'→3')**	**Amplicon (bp)**
C. maltaromaticum	F: GAGGGTCATTGGAAACTGGA R: CGGAAACCCTCCAACACTTA	219
B. thermosphacta	F: GCGCTGGATTAGCTAGTTGG R: CAGAGCTTTACGACCCGAAG	203
S. liquefaciens	F: ACGTCTACGGACCAAAGTGG R: GTGCAATATTCCCCACTGCT	191

4.3.3 DNA preparation for standard curves

Overnight cultures were prepared in BHI broth inoculated with one or two colonies from TSA. Overnight cultures were serially diluted in 10-fold increments up to 10^{-7} with sterile deionized water. One ml of each diluted culture was transferred into five Eppendorf tubes and centrifuged at 10,000 × g for 7 min. The supernatant was gently discarded and 50 µl elution buffer (Tris-HCl) from the QIAGEN microbial DNA extraction kit added to each tube. Tubes were vortexed until the cell pellet was completely resuspended. Afterward, all resuspensions were transferred into power bead tubes (micro bead tubes, bead size 0.15 mm garnet; DNeasy UltraClean Microbial Kit, QIAGEN). Bead beating was performed using a Tissue Lyser II (QIAGEN, Hilden, Germany) at 30 oscillations/s for 15 min. Tubes were centrifuged at 10,000 × g for 2 min, and clear supernatant transferred into DNase/ RNase-free sterile 2-ml collection tubes followed by heating at 100^{o}C for 10 min using a dry-heat block. After heating, the liquid preparation was again centrifuged at 10,000 × g for 2 min and the clear supernatant used as DNA template for standard curve construction. Plate counts on TSA were also performed to measure CFU/ml for each dilution.

4.3.4 Standard curves

The DNA template preparation for each isolate and each diluted culture was used in a qPCR reaction to construct standard curves for *C. maltaromaticum*, *B. thermosphacta* and *S. liquefaciens*. SYBR green-based reaction mixture (SensiFAST SYBR, NO-ROX Kit Mix, BIOLINE) was used for qPCR reactions. The composition of the 10 µl reaction mixture was 5 µl of 2 × SYBR green reaction mix, 1 µl water, 1 µl (10 µM) forward primer, 1 µl (10 µM) reverse primer, and 2 µl prepared DNA template. Reactions were performed in a Rotor-Gene Q instrument (QIAGEN, Hilden, Germany).

Cycle profiles for *C. maltaromaticum, B. thermosphacta* consisted of an initial hold at 95^{o}C for 3 min, followed by 35 cycles of denaturation at 95^{o}C for 10 s annealing of primer at 65^{o}C for 15 s, and extension at 72^{o}C for 20 s. A slight modification of the reaction mixture as well as cycling condition was used for *S. liquefaciens* to avoid primer dimer formation. Specifically, 10 µl reaction mixture of *S. liquefaciens* contained 5 µl 2 × SYBR green master mix, 0.5 µl forward primer, 0.5 µl reverse primer, 2µl water, and 2 µl DNA template. The reaction cycle included an initial hold at 95^{o}C for 5 min, then 35 cycles of holding at 95^{o}C for 10 sec, 65^{o}C for 15 sec, and extension at 72^{o}C for 20 sec. An additional step of 85^{o}C for 20 sec was added in each cycle to avoid measuring fluorescence from the primer dimer, which had a T_m of 82^{o}C.

The slope of the log-linear portion of a standard curve (bacterial concentration or gene copy versus C_t) is used to determine efficiency as per formula (Bustin et al., 2009):

PCR efficiency (E) = $10^{-1/slope} - 1$ Eq. 2

For each tested concentration, three biological replicates, containing two technical replicates, were used to generate melt curves and standard curve plots for each species.

4.3.5 Reproducibility

C_t values were generated six times for a single sample within each run (intra-run) and six times between runs (inter-run). The CV for intra- and inter-runs were calculated based on both C_t values and CFU/ml values (Table 4.2), by applying the formula:

CV = (standard deviation/mean) *100% Eq. 1

4.3.6 Evaluation of primer specificity with decreasing amount of DNA template

Each set of primers was evaluated with high to low DNA template concentrations (i.e. DNA extracted from 10^7 to 10^3 CFU/ml of overnight culture) in the presence of DNA from the other isolates to confirm no cross-isolate amplification. Standards for each isolate included a positive control.

4.3.7 Comparison of plate count and qPCR enumeration during growth

Growth curves were separately generated in BHI broth for three isolates at 25°C until growth reached stationary phase. In brief, overnight cultures of *C. maltaromaticum*, *B. thermosphacta*, and *S. liquefaciens* were prepared by transferring one or two colonies from TSA plates into BHI broth and incubating each species individually at 25°C for 16 h. Serial dilutions of overnight cultures were performed to adjust bacterial concentration to 10^3-10^4 CFU/ml based on turbidity measurements. 500 µl of a ~5 log CFU/ml inoculum was injected into 50 ml BHI broth in a heat-shrunk commercial vacuum-packaged bag (Newteq, Cryovac Inc., Sealed Air Corporation, Collinsons Vacuum Packaging, Victoria, Australia). At each time point, 5.1 ml of sample was collected; 100 µl were used for plating and 5 ml was stored at -20°C, and then DNA was extracted and 2ul quantified by qPCR as stated above in section 2.3. Growth curves were generated for three replicates for each isolate by both TSA plating and qPCR.

DMFit v3.5 (ComBase; http://www.combase.cc/tools/) software was used to fit the Baranyi and Roberts (1994) growth model to kinetic data to measure lag phase duration (LPD), growth rate (GR), and maximum population density (MPD) for plate counts. Two-tailed and one-tailed *t*-tests were used to measure significant variation in growth kinetic parameters between the two methods, for each isolate.

4.3.8 Validation of qPCR quantification for mixed culture

Plate counts were compared to qPCR results using three fixed concentrations of each species in mixed culture. Three bags per concentration containing 50 ml BHI broth were vacuum-packaged and then heat-shrunk in the commercial film at 84^{o}C. Overnight cultures of *C. maltaromaticum*, *B. thermosphacta*, and *S. liquefaciens* were diluted to concentrations ranging from 3-, 4-, 5-, and 6-log CFL/ml, mixed at equal volumes, and then injected into bags to produce different concentration for each species. Prior to injection, CFU were measured on TSA in duplicate. Bags were massaged to mix the inoculum, and then 5 ml sample removed for DNA extraction and 2ul quantified by qPCR as stated above in section 2.3, following the same qPCR protocol as for bacterial quantification.

4.4 Results and Discussion

4.4.1 Primer sets and amplification

An annealing temperature of 65^{o}C and a reduced cycle number of 25 was chosen for all primer sets based on results of the gradient PCR, to select one specific pair of primer set from five sets of primers for each of the three species, avoiding unspecific amplification. This showed the absence of PCR product formation above the annealing temperature of 65.6°C. DNA amplification from corresponding species was confirmed with PCR product sizes (~200bp) on a 1.5% agarose gel with a 50 bp ladder. Primary cross-checking of selected primer sets against DNA from different species did not show detectable amplification.

Species-specific primer pairs were generated that targeted differences in the 16S rRNA gene of isolates, a highly conserved region of the 16S rRNA gene widely used in qPCR for identification and quantification of pathogenic and spoilage bacteria (Brightwell et al., 2009;

Caro et al., 2015; de Oliveira et al., 2010; Nakayama et al., 2007; Pennacchia et al., 2009; Yost and Nattress, 2002). In a mixed-culture system, to increase specificity of primer pairs and reduce method bias, an annealing temperature of 65^{o}C was selected by temperature gradient PCR, which is the upper limit of the optimum annealing temperature range (55-65^{o}C). Previous research also observed the effect of annealing temperature and cycle number on bias during selective amplification (Ishii and Fukui, 2001). The same study indicated a preference for high annealing temperature (60^{o}C) for preferential amplification of specific microorganisms for community structure studies, which is in a good agreement with our findings. However, Sipos et al. (2007) found that a low annealing temperature (47^{o}C) did not exhibit any adverse effect on primer specific non-biased PCR product formation.

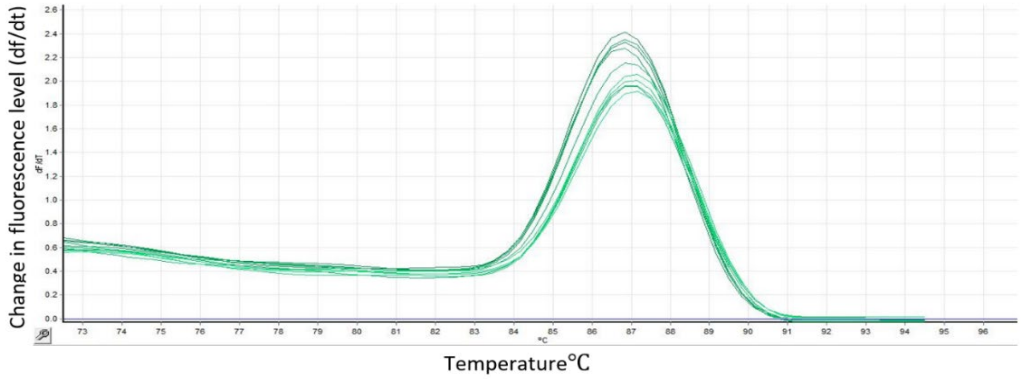
A low cycle number was suggested for the optimization step to restrict non-specific amplification or template reannealing biases and reduce side product formation (Qiu et al., 2001; Suzuki and Giovannoni, 1996). We observed a clear PCR product band with increasing annealing temperature from 60 to 65^{o}C, and by reducing cycle number from 35 to 25 cycles.

4.4.2 Specificity

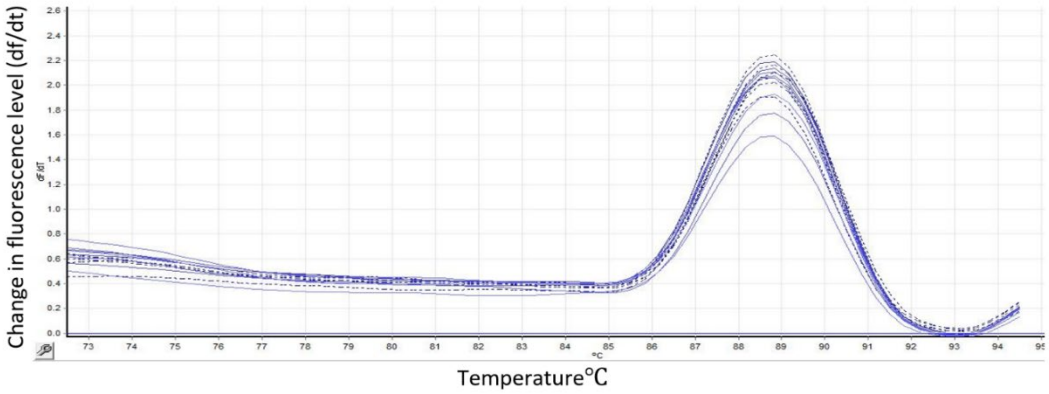
A single peak in the standard melt curve for each species demonstrated specificity of reaction (Figure 4.1, A-C). Primer dimer formation was identified during standard curve construction of *S. liquefaciens*, which was reduced by altering qPCR cycle profile and reaction mixture. Powell et al. (2006) reported a similar occurrence that is a drawback in detection chemistry of the SYBR green method. Melt curve analysis is therefore crucial to identify non-specific and false-positive amplification. In our study, secondary cross-checking

of primers against high to low concentrations of DNA from different species did not show detectable cross amplification, whereas DNA standards amplified with the corresponding primer set for each species.

A



B



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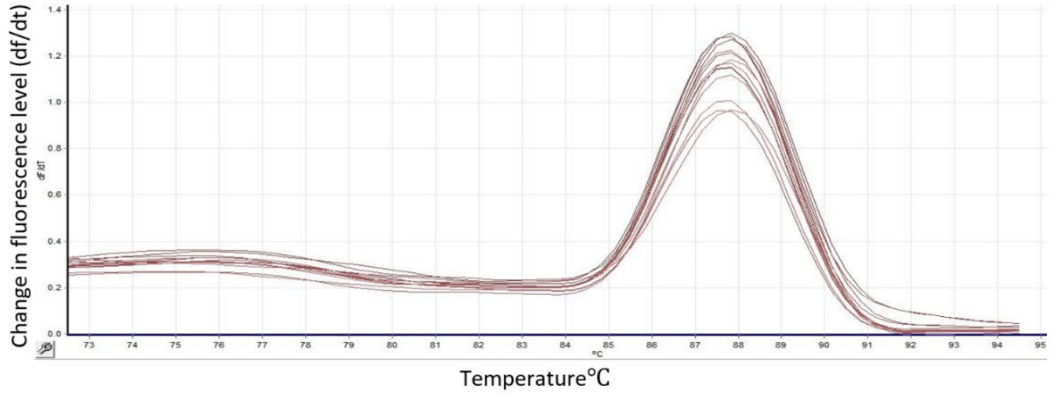


Figure 4.1. Melt curves for *C. maltaromaticum* (A), *B. thermosphacta* (B) and *S. liquefaciens* (C). Lines in each graph represent experimental runs at different tested concentrations, with two technical replicates per concentration.

4.4.3 Standard curve and detection limit (sensitivity)

Absolute quantification of a target gene requires construction of a standard or calibration curve (Svec et al., 2015). Well-constructed standard curves for qPCR rely on robustness of the PCR (amplification efficiency), dynamic range determination, limit of detection, and actual quantification (Bustin et al., 2009; Svec et al., 2015).

The reaction efficiency of the standard curves (Figure 4.2, A- C) of *C. maltaromaticum, B. thermosphacta* and *S. liquefaciens* was 1.005 (R^2 = 0.996), 1.0029 (R^2 = 0.997), and 1.005 (R^2 = 0.983), respectively. The linear range of primer set for both *B. thermosphacta and C. maltaromaticum* was 10^3 to 10^7 CFU/ml and for *S. liquefaciens* 10^4 to 10^8 CFU/ml. This protocol has not been optimized for meat matrices, however these linear ranges are within expected bacterial levels observed in stored commercial meat, in which concentrations can range from 10^2 to 10^7/g-cm^2.

An amplification efficiency 1.00 (100%) demonstrates optimum duplication of the target gene at the end of each cycle, assuming no interference. However, the acceptable range may vary between 90 to 105% (Johnson et al., 2013). In our study, standard curve efficiencies were within this range (approximately 100%), which was reflected in C_t value (i.e. 3.3 increase for 10-fold increment of cell concentration). Linear quantification over the range of a 5-log concentration (3 to 7 log CFU/ml for *C. maltaromaticum* and *B. thermosphacta*; 4 log CFU/ml to 8 log CFU/ml for *S. liquefaciens*) was observed; similar findings have been reported by (Hein et al., 2005; Martínez-Blanch et al., 2009).

A change in cycling parameter and reaction mixture was required to eliminate primer dimer formation for *S. liquefaciens.* Linear range of detection for the species were determined

experimentally considering reaction efficiency and may not be the same for different assays. qPCR primers were designed based on the 16s rRNA sequences of each species which are unique. In addition, *S. liquefaciens* is a gram-negative isolate, whereas *C. maltaromaticum* and *B. thermosphacta* are gram-positive. Such variations might have impacted linear range of detection for *S. liquefaciens*.

4.4.4 Reproducibility

The CV values, considering both bacterial concentration (CFU/ml) and thermocycler, generated C_t values for inter- or intra-run, were within 14% (Table 4.2). Minimum Information for publication of Quantitative Real-Time PCR Experiments (MIQE) guideline by Bustin et al. (2009) cited Schmittgen and Livak (2008) who recommend to consider concentration or copy number and avoid C_t values to generate CVs. A previous research report found significant variation between CVs generated based on target gene copy number and C_t value (Dionisi and Hawkins, 2003), which is also reflected in this study. CV values generated from both C_t and bacterial concentration are shown in Table 4.2 for comparison. Research by Powell et al. (2006) also preferred bacterial concentration rather than C_t values to calculate CVs. Intra-run CV was always lower than inter-run CV, whether CV was based on bacterial concentration or C_t values, similar to the findings by Powell et al. (2006) and Dionisi and Hawkins (2003).

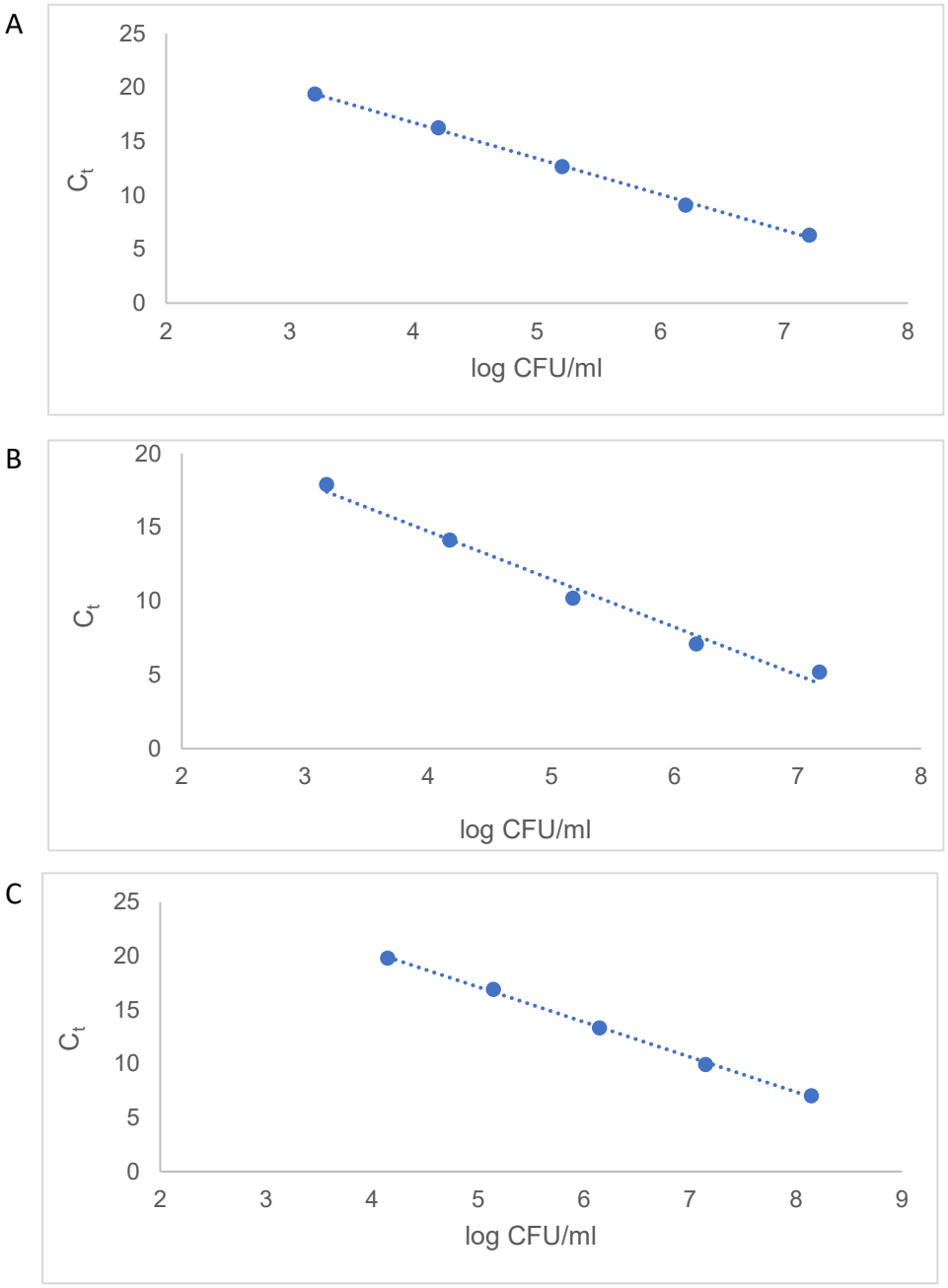


Figure 4.2. Standard curves for *C. maltaromaticum* (A), *B. thermosphacta* (B) and *S. liquefaciens* (C). For each tested concentration, three biological replicates, containing two technical replicates, were used to generate melt curves and standard curve plots for each species.

Table 4.2. CV values based on bacterial concentration and C_t value for three isolates

Species	CV based on CFU/ml [1]		CV based on C_t	
	intra-run (%)	inter-run (%)	intra-run (%)	inter-run (%)
C. maltaromaticum	3.54	8.87	0.40	8.09
B. thermosphacta	4.61	11.25	0.43	9.85
S. liquefaciens	13.01	13.80	1.11	9.11

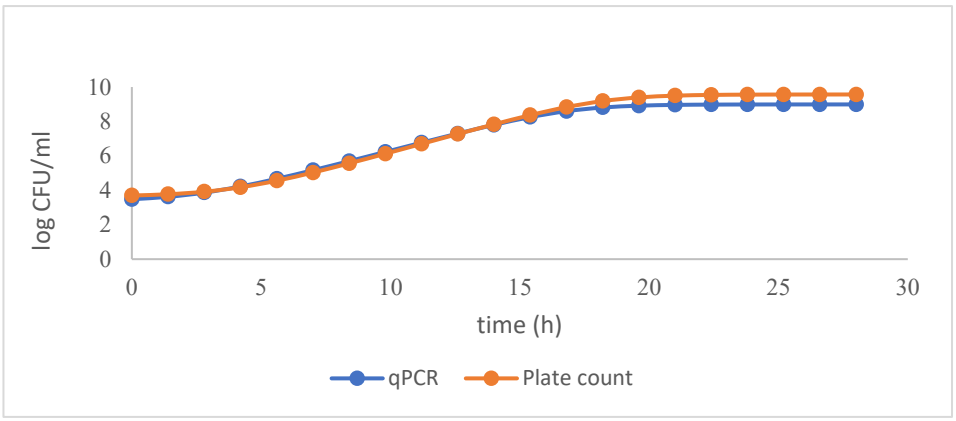
[1]six replicates were used to generate CV values.

4.4.5 Comparison between qPCR and plate count data

Growth curves (Figure 4.3) were determined by fitting the Baranyi and Roberts (1994) model to kinetic data obtained from both plate count and qPCR methods. LPD was not considered due to high innate variation among replicates.

Although mean MPD by qPCR was lower (8.83 CFU/ml) compared to plate count method (9.12 CFU/ml), the difference was not significant (p-value = 0.10, two-tailed test). Mean values for GR measured by qPCR (0.389 CFU/h) and plate count (0.388 CFU/h) were also not significantly different (p-value= 0.97, two-tailed test). Similar findings were reported by Reichert-Schwillinsky et al. (2009) who found maximum GR as well as cell counts of *L. monocytogenes* under optimum growth conditions were identical between qPCR and plate count methods. Guan et al. (2017) also reported that predictive model constructed for *Staphylococcus aureus* based on plate count data and qPCR data were not significantly

A



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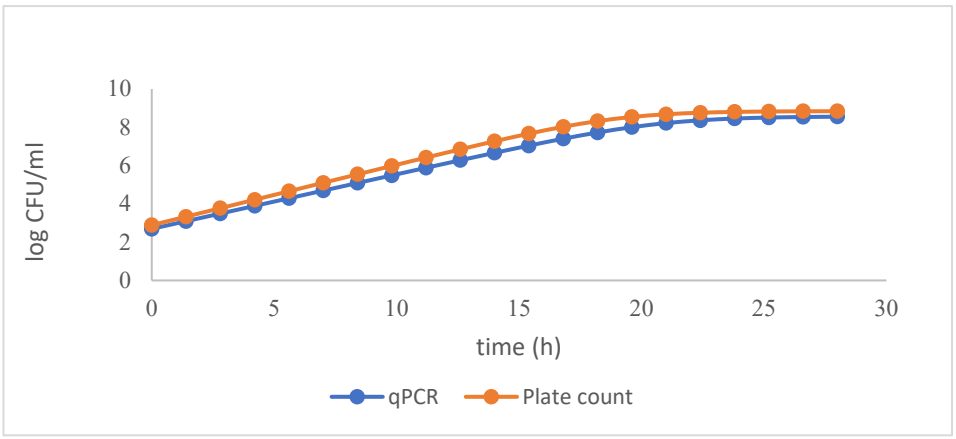

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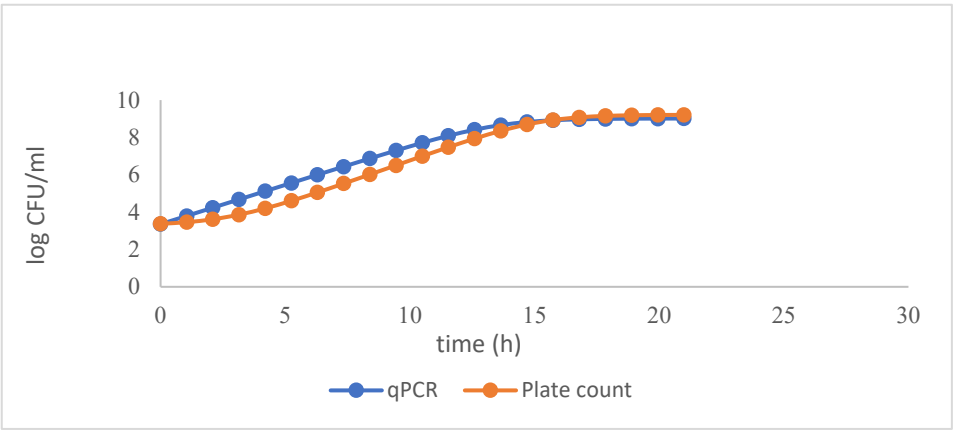


Figure 4.3. Growth curves for qPCR and plate count methods for *C. maltaromaticum* (A), *B. thermosphacta* (B) and *S. liquefaciens* (C) at 25°C

Table 4.3. Comparison between bacterial quantification by pure culture on agar plates and mixed culture by qPCR. Target levels for sample bags were: bag 1, 4 log CFU/ml *C. maltaromaticum,* 3 log CFU/ml *B. thermosphacta* and 4 log CFU/ml *S. liquefaciens*; bag 2, 5 log CFU/ml *C. maltaromaticum,* 4 log CFU/ml *B. thermosphacta* and 5 log CFU/ml *S. liquefaciens*; bag 3 contained 6 log CFU/ml *C. maltaromaticum,* 5 log CFU/ml *B. thermosphacta* and 4 log CFU/ml *S. liquefaciens.*

Species	Sample	Plate count (log CFU/ml)	qPCR (log CFU/ml)
C. maltaromaticum	1	4.05	3.93
	1	4.05	3.97
	2	5.05	4.97
	2	5.05	4.84
	3	6.05	5.96
	3	6.05	5.99
B. thermosphacta	1	3.01	3.00
	1	3.01	3.06
	2	4.01	4.07
	2	4.01	4.12
	3	5.01	5.09
	3	5.01	5.00
S. liquefaciens	1	4.59	4.66
	1	4.59	4.44
	2	5.59	5.42
	2	5.59	5.71
	3	4.59	4.50
	3	4.59	4.87

different, in agreement with other researchers investigating similarities between conventional plate count data and detection threshold of qPCR (Alarcon et al., 2006; Chen et al., 2010; Hein et al., 2001; Hierro et al., 2007; Perelle et al., 2004; Takahashi et al., 2009).

4.4.6 Validation of qPCR quantification

There was negligible variation between qPCR and plate count measurements for each species (Table 4.3; $R^2 = 0.98$) at broth inoculum levels ranging from approximately 3 to 6 log CFU/ml. Specifically, 0.06 to 0.21, -0.11 to 0.01, and -0.28 to 0.17 log CFU/ml variation was observed between plate count and qPCR for *C. maltaromaticum, B. thermosphacta* and *S. liquefaciens*, respectively. Based on growth curve kinetics, there were insignificant differences for GR and MPD between plate count and qPCR methods.

4.5 Conclusions

Mixed-species experimental systems must be used to study the evolution of spoilage microbiomes, including quantifying growth dynamics of individual species within the community. This poses a difficult problem, considering the many limitations associated with traditional bacterial culture.

Quantitative descriptions of bacterial growth kinetics must be initially conducted in a highly defined matrix (e.g. microbiological broth), in which is it possible to control environmental and bacterial variables. We anticipate that the developed qPCR method will be a valuable tool to understand bacterial growth kinetics and inter-species interactions under intrinsic environmental conditions relevant to meat (e.g. as pH, lactic acid, glucose). Further experiments are required to validate this qPCR protocol for other matrices and in the presence of other microbiota.

We report a 16S rRNA gene-based qPCR technique that enables selective amplification and accurate quantification of single bacterial species within a mixed culture system. This method can facilitate quantification of bacterial growth kinetics and development of predictive models, which may result in new processing interventions to improve food safety and reduce spoilage and waste.

Chapter 5. Effect of Undissociated Lactic Acid on Growth Kinetics and Interactions of *Carnobacterium maltaromaticum, Brochothrix thermosphacta* and *Serratia liquefaciens* in Mixed Culture

5.1 Abstract

The effect of undissociated lactic acid (UDLA), generated from two pH levels (5.5 and 6.5) and 5 lactic acid concentrations (0, 25, 50, 75, 100 mM), on the growth of three major meat-associated microorganisms was investigated using a simulated beef matrix vacuum-packaged (VP) in a commercial heat-shrunk film and stored at 10^{o}C. A 16S rRNA gene-targeted SYBR green-based qPCR method was used to quantify bacterial growth in individual-, 2- and 3-species mixed cultures. Growth only occurred at up to 1.68 mM UDLA (75 mM lactic acid at pH 5.5) regardless of species or culture combinations. For *Brochothrix thermosphacta*, growth rate (GR) was marginally reduced at 0.56 to 1.68 mM UDLA (p-value=0.046), however maximum population density (MPD) decreased significantly when UDLA increased from 0.06 to 1.68 mM UDLA (p- value < 0.05) in the 3-species mixed culture. *B. thermosphacta* MPD inhibition in 3-species mixed cultures increased gradually as UDLA increased. The 2-species cultures revealed that *Carnobacterium maltaromaticum* produced greater inhibition of *B. thermosphacta* MPD compared to *Serratia liquefaciens*, at 0.11, 0.17, 0.22 and 1.12 mM UDLA concentrations. In 3-species mixed culture, *C. maltaromaticum* GR and MPD were only marginally altered relative to monoculture at most UDLA levels. A large difference was however observed at 1.68 mM UDLA for MPD in which *C. maltaromaticum* is able to sustain a high population in the mixed culture unlike monocultures where MPD was suppressed. At 2.24 mM UDLA this difference was

abolished due to growth inhibition. UDLA did not affect the growth of *S. liquefaciens* (p-value=>0.14) in the 3-species mixed culture. Species sensitivity and bacterial interactions at 0 to 1.68 mM UDLA occurred in the order *B. thermosphacta > C. maltaromaticum > S. liquefaciens*. The 'Jameson effect' may have caused the reduction in *B. thermosphacta* MPD in 2-species co-cultures with *C. maltaromaticum*, in contrast to MPD reduction observed for *S. liquefaciens*. An acceptable accuracy factor 1.12 for all three species indicated the validation of mBHI results in commercial VP beef. The effects of UDLA on microbial interactions demonstrated here could be utilised by processors in a more targeted way to control spoilage of highly perishable muscle foods, such as VP beef.

5.2 Introduction

The meat industry suffers economic loss when consumers reject products due to undesirable textural or chemical changes that produce off-odours, off-flavours, slime, or discoloration (Gram et al., 2002; Jackson eta al., 1992). Such spoilage can be caused by microbial growth/metabolism, lipid oxidation, and/or enzymatic degradation (Dave and Ghaly, 2011). Of these factors, less is understood about the mechanisms of microbial spoilage, specifically factors that drive the evolution of multi-species spoilage microbiomes.

Extrinsic (e.g. temperature, atmosphere) and intrinsic (e.g. pH, water activity, lactic acid, glucose) factors influence species growth and survival within a meat microbiome (Borch et al., 1996). Among these, pH and lactic acid (LA) strongly influence bacterial growth rate (Grau, 1980, 1981; Greer and Dilts, 1995; Janssen et al., 2007). This illustrated in Chapter 3 of this thesis which examined the effect of glucose, pH and LA on *C. maltaromaticum, B. thermosphacta* and *S. liquefaciens* within a commercial heat-shrunk VP film. A pH range of 5.5

to 6.5 is typical of post-mortem beef, in which higher pH produces undesirable a dark, firm, and dry (DFD) texture (Gill and Newton, 1982). LA varies between 90 to 100 mM, formed by metabolism of muscle tissue glycogen (Garcia-Lopez et al., 1998), which influences pH. Consequently, higher muscle glycogen reserves are preferred in processed meat, as they maintain pH <6 and survival of lactic acid bacteria (LAB), in contrast to DFD meat that favours growth of undesirable species (Gill and Newton, 1982).

LA inhibits growth of bacteria via its undissociated form (UDLA), which traverses the bacterial cell membrane, lowers intracellular pH, and disrupts cell metabolism (Gill, 1986; Gill and Newton, 1982; Grau, 1980). LAB tolerate relatively high UDLA levels and moderately acidic pH. This phenomenon is well recognized by meat processors, however the mechanisms that drive the evolution of desirable microbial communities are poorly understood.

C. maltaromaticum, B. thermosphacta, and *S. liquefaciens* are dominant bacterial species found in VP refrigerated beef spoilage community (Ercolini et al., 2009; Ercolini et al., 2006; Nychas et al., 2008). *B. thermosphacta* and *S. liquefaciens* produce off-odours caused by metabolites (e.g. acetoin) and H_2S, respectively (Casaburi et al., 2014; Patterson and Gibbs, 1977). In contrast, *C. maltaromaticum* is a preferred LAB species (Jones, 2004; Laursen et al., 2005) because it is associated with higher sensory scores, including VP beef primals refrigerated for up to 30 weeks (Kaur et al., 2017a; Small et al., 2012).

Synergism-cooperation and antagonism-competition within mixed bacterial populations influence how spoilage species survive and dominate spoilage microbiomes (Cotto et al., 2015; Gram et al., 2002). Yet we know very little about inter-species interactions and associated environmental effects, compared to individual-culture studies that have commonly investigated

a single species, but sometimes two species (Zhang et al. 2015; Cotto et al., 2015; Gill, 1986). Historically, a major reason is the lack of discriminating methods to quantify the growth of individual species within mixed populations (Hernandez-Macedo et al., 2011). However, recent advancements in molecular techniques (e.g. qPCR) overcome many of these challenges.

Species-specific qPCR methods that target highly conserved regions of the 16S rRNA gene allow quantification of individual species kinetics within microbial communities (Cotto et al., 2015). The copy number of the 16Sr RNA gene can be interpreted as biomass or bacterial concentration, without the need for isolation or enrichment (Cotto et al., 2015; Reichert-Schwillinsky et al., 2009). Although qPCR has been developed for species found in VP beef, such as *Carnobacterium* spp., *B. thermosphacta* and *Serratia* spp. (Pennacchia et al., 2009; Takahashi et al., 2017; Yost and Nattress, 2002), there are no published reports where qPCR has been used to measure *C. maltaromaticum, B. thermosphacta,* and *S. liquefaciens* growth kinetics within a mixed culture system, except as shown in Chapter 4 of this thesis. The study presented here quantifies the effect of UDLA on individual growth kinetic parameters of *C. maltaromaticum, B. thermosphacta*, and *S. liquefaciens* in mixed culture.

5.3 Materials and Methods

5.3.1 Experimental design

Individual growth kinetics of *C. maltaromaticum* (C0a), *B. thermosphacta* (A8f), and *S. liquefaciens* (D0d) were studied in 1-, 2-, and 3-species culture systems. The first experiments performed were 1- and 3-species systems; if differences in growth kinetics were observed for an individual species, then 2-species studies were conducted to determine the species that inhibited or promoted GR and/or MPD.

LA was adjusted to 0, 25, 50, 75 and 100 mM, at pH 5.5 and 6.5, using a full factorial design of 10 (5x2) media formulations, and the whole growth experiment was conducted in two separate trials (Table 5.1); two VP bags of each formulated media were used per trial. Lag phase duration (LPD), GR and MPD were measured.

5.3.2 Bacterial strains and inoculum

Chapter 3 describes source, maintenance, and subculture of *C. maltaromaticum* (C0a), *B. thermosphacta* (A8f), and *S. liquefaciens* (D0d). In brief, they were originally obtained from VP beef primals sourced from six different Australian abattoirs. Frozen isolates were subcultured on Tryptone Soya Agar (TSA; Oxoid, Thebarton, Australia), and then in sterile Brain Heat Infusion broth (BHI; Amyl Media Ltd., Melbourne, Australia) at 25^{o}C for 16 h. Each culture was diluted with sterile BHI broth for an inoculum of 10^5 CFU/ml.

5.3.3 Media formulation

General procedure for preparing modified brain heart infusion (mBHI) broth is described in Chapter 3, except that here LA was adjusted to 0, 25, 50, 75, and 100 mM, at pH 5.5 and 6.5 (Table 5.1). UDLA concentration was calculated by the formula described in Chapter 3 (*Media formulation* section of Materials and Methods).

Table 5.1. Modified brain heart infusion (mBHI) broth formulations

Medium	pH	LA (mM)	UDLA (mM)
1	6.5	0	0.00
2	6.5	25	0.06
3	6.5	50	0.11
4	6.5	75	0.17
5	6.5	100	0.22
6	5.5	0	0.00
7	5.5	25	0.56
8	5.5	50	1.12
9	5.5	75	1.68
10	5.5	100	2.24

5.3.4 Preparation of media in vacuum-sealed heat-shrunk bags

50 ml of formulated sterile broth was vacuum-sealed and heat-shrunk in commercial vacuum barrier film bags, as described in Chapter 3 (*Preparation of media in commercial vacuum-sealed shrink bags* section of Materials and Methods). The surface of bags was wiped with 70% ethanol, and a small piece of latex sealing tape (Stylus Tapes International, Victoria, Australia) was attached to facilitate injecting inocula and withdrawing samples with a sterile syringe. Broth sterility was verified by incubating bags at room temperature (~25^{o}C) overnight and plating 100 µL broth on TSA.

5.3.5 Sample inoculation and collection

500 µl of inoculum (1-, 2- and 3-species) was injected into bags, which were then manually massaged; 5.1 ml was immediately removed by syringe. Of this volume, 5.0 ml was used for qPCR analyses and 0.1 ml (100 µl) to enumerate colonies on TSA. Bags were incubated at 10˚C; 5 ml of broth was removed by syringe at each sampling time (over 80 to 1100 h), transferred to five 1.5 ml sterile tube, and stored at -20°C.

5.3.6 qPCR

DNA was extracted from the 5 ml stored samples, following the optimized method for standard curve construction described in Chapter 4 (*DNA preparation for standard curves* section of Materials and Methods), and then stored at -20°C until qPCR was performed. 2 µl was added as template DNA to an 8 µl reaction mixture (5 µl SYBR green based reaction mixture, 1 µl (10 µM) forward primer, 1 µl (10 µM) reverse primer, 1 µl deionized nuclease-free water) for a final volume of 10 µl. Selected primer sets for each species, as described in Chapter 4 (*Primer design and selection of primer set* section of Materials and Methods), were used in reaction mixtures; standard curve samples were included for each run. Cycling conditions were as described in Chapter 4 (*Standard curves* section of Materials and Methods). A total of 150 growth curves (30 individual + 30 3-species culture + 15 2-species) were generated among all experiments.

5.3.7 Validation of mBHI growth kinetics in VP beef

Growth kinetics observed in mBHI were compared to those observed in irradiated commercial VP beef. Bacterial cultures and inocula were prepared as described above.

A whole beef striploin primal (~5 kg) was purchased from a local retail store in Tasmania, Australia. The primal was cut into 350 pieces of ca. 5 cm x 5 cm x 1 cm, using a sterile knife, with

each piece weighing 15 ± 5 g. Individual pieces were transferred into sterile commercial vacuum barrier shrink bags and vacuum-packaged following the procedures described in Chapter 3 (*Preparation of media in commercial vacuum-sealed shrink bags* section of Materials and Methods). Meat samples were kept in the refrigerator at 2^{o}C for 2 d, shipped by overnight carrier at 2 to 4^{o}C to Steritech Pty. Ltd, Victoria, Australia, and there sterilized by irradiation at 10 kGy. Following irradiation, treated samples were refrigerated and shipped back to the laboratory overnight. Upon arrival, samples were stored at 2^{o}C until experimentation. Three packs were randomly selected to test sterility. After overnight incubation at 25^{o}C, 10 ml sterile peptone water was added to each of three packs which were manually massaged, and then 100 µL plated on BHI agar. Plates were incubated at 25^{o}C for 48 h. Prior to experimentation, the outer surface of each pack was wiped with 70% ethanol, and then a small piece of latex sealing tape adhered to the outside of the pack. The septum was subsequently used to inject the inoculum.

pH was measured for three randomly selected pieces of irradiated meat. In a separate experiment, meat pH was increased to 6.5 by adding 50 µL of filter-sterilised NaOH (1M) to the meat surface, and then equilibrated for one hour prior to experimentation and inoculation. Meat lactic acid and glucose levels were measured by a commercial food testing laboratory (DTS Food Assurance, Victoria, Australia).

A total of 180 meat samples (15-time points × 3 bacterial species × 2 replicates × 2 pH levels) plus a negative control were individually inoculated with one of the three isolates. Prior to inoculation, each pack was weighed, the outer surface of the septum wiped with 70% ethanol,

and then 100 µL of inoculum (2-3 $\log_{10}$ CFU/ml) injected onto the meat surface. Inoculated samples were incubated at 10°C for eight to 24 d, depending on species and pH treatment.

Bacterial counts were measured in two packs of inoculated meat at each sampling interval. Packs were opened with sterile scissors, 10 ml sterile peptone water added, and content transferred to a filter stomacher bag and stomached for 30 sec. Afterward, 100 µL was direct-plated on BHI agar; another 100 µL was transferred to a micro-centrifuge tube containing 900 µL BHI broth, serially diluted in 10-fold increments, and plated on BHI agar. Plates were incubated at 25°C for 48 h for *B. thermosphacta* and *C. maltaromaticum*, and for 24 h at 25°C for *S. liquefaciens*. Colonies were counted and recorded as $\log_{10}$ CFU/g.

5.3.8 Data analysis

The Baranyi and Roberts growth model was fitted to kinetic data to estimate LPD, GR, and MPD, using DMFit software (v3.5 [ComBase; http://www.combase.cc/tools/]). The General Linear Model (GLM) in SAS (v 9.4; SAS, Inc., Rockville, MD, USA) was used to compare biological replicates (trials) and average GR and MPD for individual versus 3-species culture systems at all LA levels and pH; significance was not measured for 2-species experiments. A p-value <0.05 was considered significant. GRs observed in beef were compared to observations in mBHI broth (Chapter 3) by applying a linear regression to the latter data and then estimating GR at the LA level observed in beef. Bias (B_f) and accuracy (A_f) factors and acceptable predictions were calculated by the methods of Ross (1996) and Oscar (2005).

5.4 Results

LPD showed high variation and no significant correlation among all treatments (Supplemental tables). The following results describe the effects of UDLA on GR and MPD, in which growth

kinetics for a single species were compared to the same species in a 3-species culture of *B. thermosphacta*, *C. maltaromaticum*, and *S. liquefaciens*. If significant differences in GR or MPD were observed between individual and the 3-species culture, then the target species was cultured separately in 2-species mixtures to determine the source(s) of effect.

5.4.1 Brochothrix thermosphacta

Growth rate

At 0 to 0.22 mM UDLA (pH 6.5), there was no significant difference or correlation between the average overall GR of *B. thermosphacta* in individual and 3-species culture (Figure 5.1 and 5.2; Supplemental Table A.1; Supplemental Figure A.1-A.4). In contrast, at 0.56 and 1.12 mM UDLA (pH 5.5), the average individual GR was significantly higher than in the 3-species mixture (Figure 5.1 and 5.2; Supplemental Table A.2; Supplemental Figure A.5-A.8), which corresponded to GR differences of 0.007 and 0.011 log CFU/h at 0.56 and 1.12 mM UDLA, respectively. No growth was observed at 1.68 or 2.24 mM UDLA.

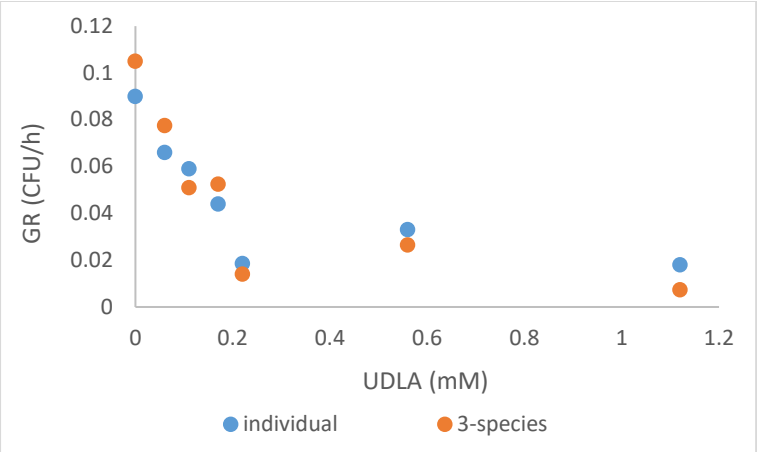


Figure 5.1. *B. thermosphacta* GR as a function of undissociated lactic acid (UDLA) in individual versus 3-species culture.

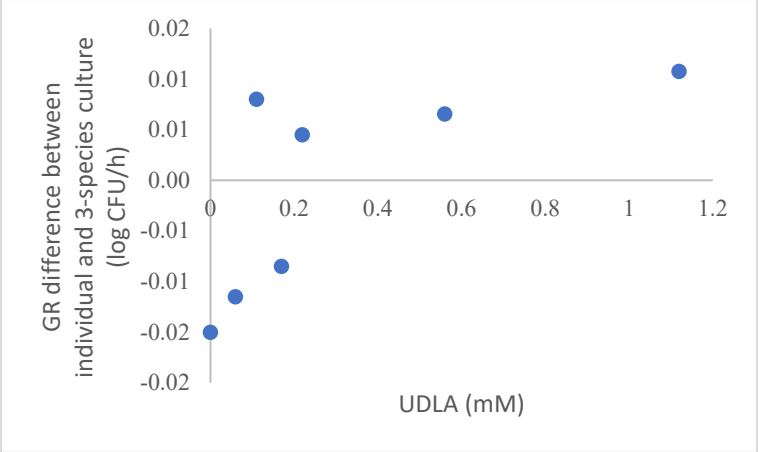


Figure 5.2. Difference in GR for individual and 3-species culture versus UDLA concentration for *B. thermosphacta*.

A 2-species co-culture was only evaluated if there was a significance difference in average GR or MPD when comparing individual and 3-species cultures. Culturing *B. thermosphacta* separately with *C. maltaromaticum* and *S. liquefaciens* revealed that the former species exerted a greater inhibitory effect on *B. thermosphacta* GR, such as at 1.12 mM UDLA (i.e. 0.016 versus 0.007, respectively; Supplemental Table A.2; Supplemental Figure A.6). The sum of each 2-species effects on GR was 0.023 log CFU/h, which was greater than the effect observed in the 3-species culture.

Maximum population density

At 0 to 0.22 mM UDLA (pH 6.5), a significant difference and correlation between the average MPD of *B. thermosphacta* in individual and 3-species culture was observed (Figure 5.3 and 5.4; Supplemental Table A.1; Supplemental Figure A.1-A.4), although such trend was not observed at >0.22 mM UDLA. Similarly, a significantly higher MPD was observed in individual vs 3-species culture at 0.56 and 1.12 mM UDLA (Figure 5.3 and 5.4; Supplemental Table A.2; Supplemental Figure A.5-A.6), which corresponded to MPD differences of 0.84 and 2.52 log CFU/ml at 0.56

and 1.12 mM UDLA, respectively. No growth was observed at 1.68 or 2.24 mM UDLA.

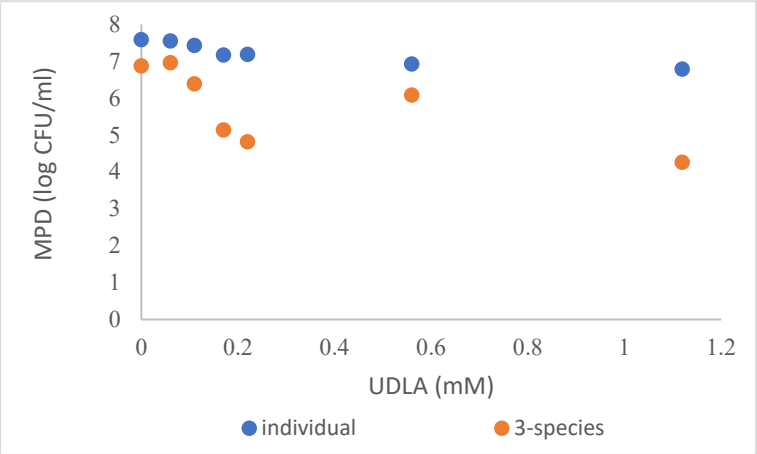


Figure 5.3. *B. thermosphacta* maximum population density (MPD) as a function of UDLA in individual versus 3-species culture.

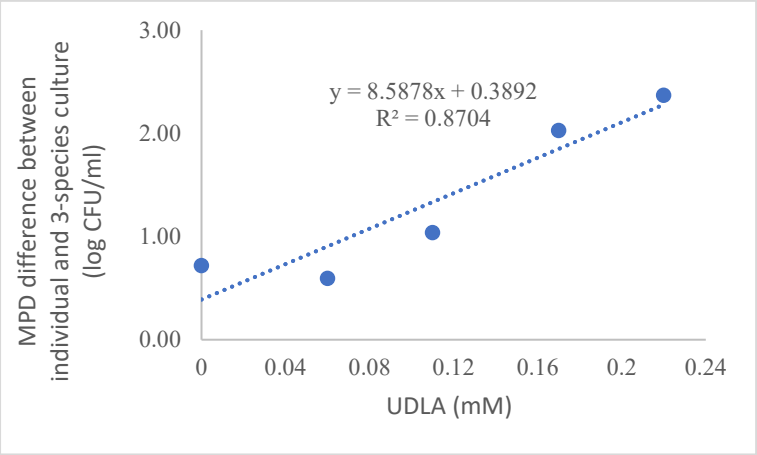


Figure 5.4. Difference in *B. thermosphacta* MPD for individual and 3-species culture as a function of UDLA from 0 to 0.22 mM.

When separately cultured with *C. maltaromaticum* and *S. liquefaciens* at 0.11 mM UDLA, the former species accounted for the majority of MPD inhibition (1.14 versus 0.58 log CFU/ml, respectively) of *B. thermosphacta* observed in the 3-species culture (1.04 log CFU) (Supplemental Table A.1, Supplemental Figure A.2). The inhibitory effect of *C. maltaromaticum*

on *B. thermosphacta* MPD increased at 0.17 and 0.22 mM UDLA, similar to the effect of *S. liquefaciens*. At >0.22 mM UDLA, when *B. thermosphacta* was grown separately with *C. maltaromaticum* and *S. liquefaciens*, both species strongly inhibited *B. thermosphacta* MPD, i.e. 3.59 and 2.18 log CFU/ml, respectively, at 1.12 mM UDLA (Supplemental Table A.2, Supplemental Figure A.6). The sum of the 2-species effects on MPD was 5.77 log CFU/ml, which was greater than the effect observed in 3-species culture.

5.4.2 Carnobacterium maltaromaticum

Growth rate

At 0 to 0.22 mM UDLA (pH 6.5), there was no significant difference or correlation between the average overall GR of *C. maltaromaticum* in individual and 3-species culture (Figure 5.5 and 5.6; Supplemental Table B.1; Supplemental Figure B.1-B.4). In contrast, at 0.56, 1.12 and 1.68 mM UDLA (pH 5.5), the average GR in 3-species culture was significantly higher than the individual GR (Figure 5.5 and 6; Supplemental Table B.2; Supplemental Figure B.5- B.7). Similar to *B. thermosphacta*, *C. maltaromaticum* did not grow at 2.24 mM UDLA in individual culture or 3-species culture. However, an exception was observed at 1.68 mM in which it grew in the 3-species culture with GR 0.015 CFU/h but not in individual culture (Figure 5.5, Supplemental Table B.2, Supplemental Figure B.7).

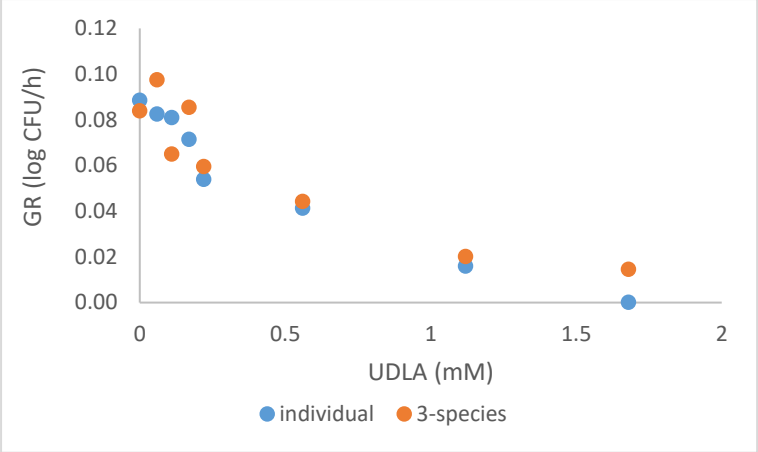


Figure 5.5. *C. maltaromaticum* GR as a function of UDLA in individual versus 3-species culture.

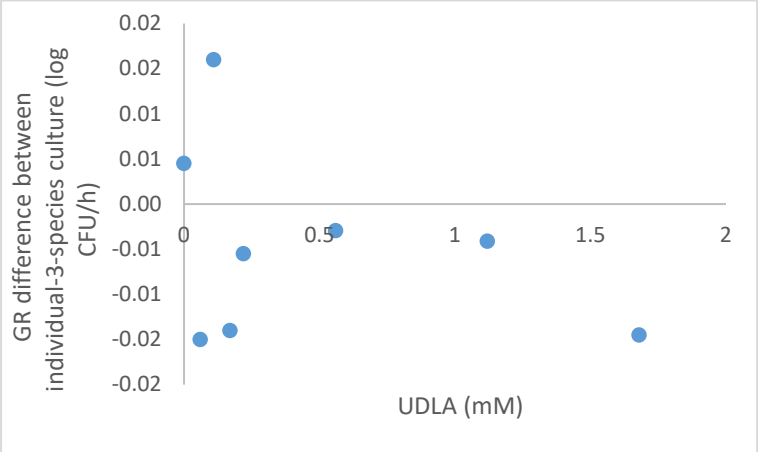


Figure 5.6. Difference in *C. maltaromaticum* GR for individual and 3-species culture as a function of UDLA concentration.

When *C. maltaromaticum* was grown separately with *B. thermosphacta* and *S. liquefaciens*, there was little effect (<0.008 log CFU/h) on *C. maltaromaticum* GR for any UDLA level (Supplemental Table B.1 and B.2).

Maximum population density

From 0 to 0.22 mM UDLA (pH 6.5), the average MPD for *C. maltaromaticum* was significantly higher in the individual versus the 3-species culture (Figure 5.7 and 8; Supplemental Table B.1;

Supplemental Figure B.1-B.4); however, there was no significant difference at 0.56 and 1.12 mM UDLA (pH 5.5). At 1.68 mM UDLA, *C. maltaromaticum* reached 7 log CFU/ml in the 3-species culture but not when grown individually for 1000 h (Figure 5.7; Supplemental Table B.2; Supplemental Figure B.7), corresponding to MPD differences of 4.81 log CFU/ml. No growth was observed at 2.24 mM UDLA. Unlike *B. thermosphacta* (Figure 5.4), for *C. maltaromaticum* the difference between individual and 3-species MPD did not progressively decrease as UDLA increased. Instead, the inhibitory effect of UDLA decreased with increasing UDLA up to 0.22 mM UDLA (Figure 5.8), which was the opposite effect observed for *B. thermosphacta*.

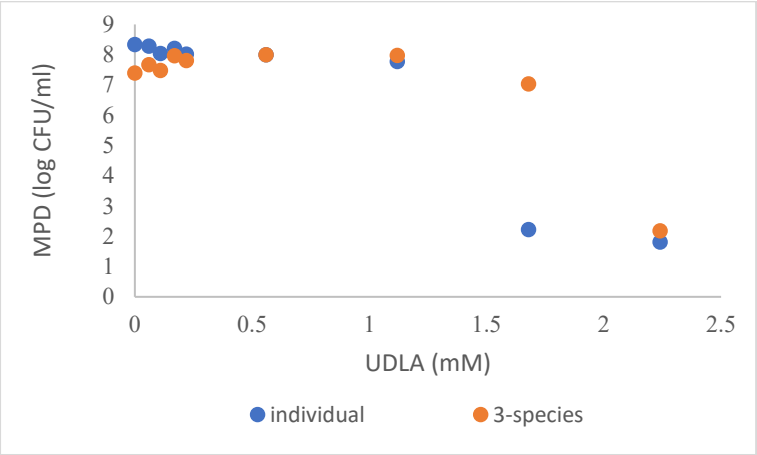


Figure 5.7. *C. maltaromaticum* MPD as a function of UDLA in individual versus the 3-species culture.

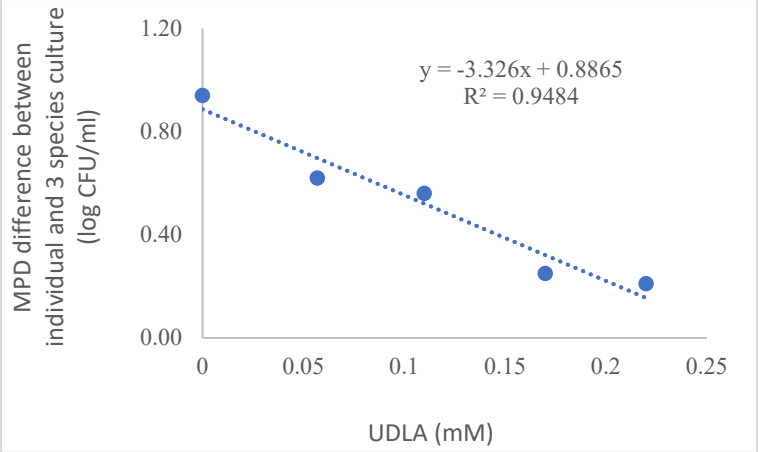


Figure 5.8. Difference in MPD for individual and 3-species culture versus UDLA concentration (0 to 2.24 mM) for *C. maltaromaticum*.

When separately cultured with *B. thermosphacta* and *S. liquefaciens*, there was little effect (i.e. <0.3 log CFU) on *C. maltaromaticum* MPD for any UDLA level (Supplemental Table B.1 and B.2). Also, neither species promoted the growth of *C. maltaromaticum* when separately cultured with *B. thermosphacta* and *S. liquefaciens* (Supplemental Figure B.7).

5.4.3 Serratia liquefaciens

Growth rate

Up to 1.68 mM UDLA, there was no significant difference or correlation between GR of *S. liquefaciens* in individual and 3-species mixed culture (Figure 5.9 and 5.10). Growth was not observed at 2.24 mM UDLA. No trend was observed in GR difference between individual and 3-species culture versus UDLA concentration (Figure 5.10).

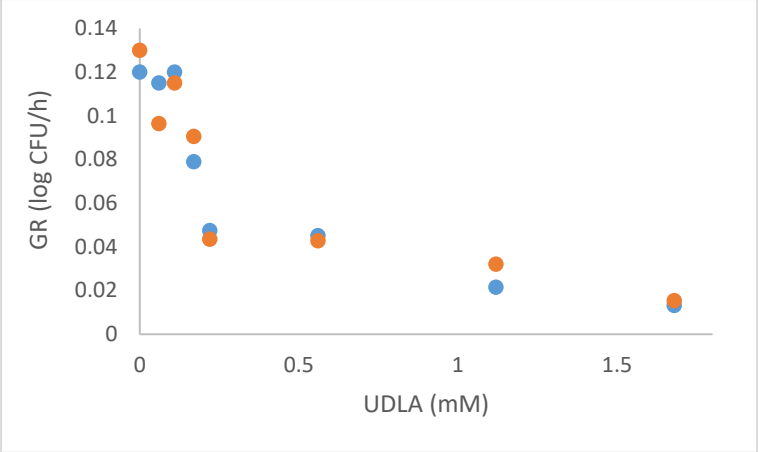


Figure 5.9. *S. liquefaciens* GR as a function of UDLA in individual versus 3-species culture.

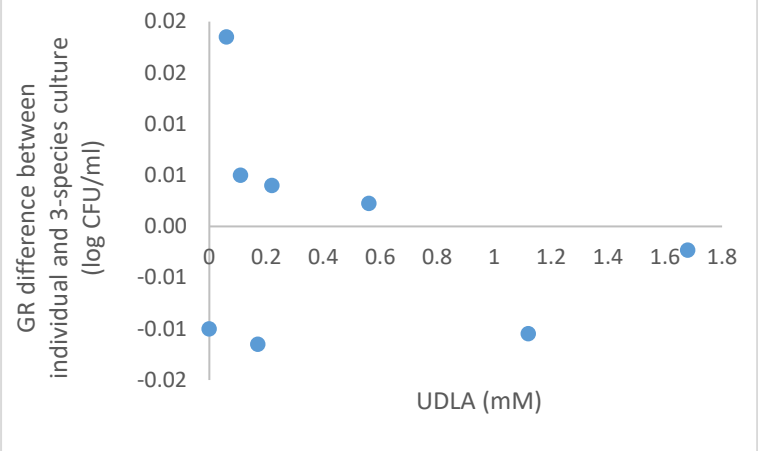


Figure 5.10. Difference in *C. maltaromaticum* GR for individual and 3-species culture as a function of UDLA concentration.

In 2-species culture at 1.12 mM UDLA, *C. maltaromaticum* and *B. thermosphacta* produced no effect on *S. liquefaciens* GR compared to the 3-species culture (Supplemental Figure C.6). However, at 1.68 mM UDLA, *S. liquefaciens* did not grow when co-cultured with *C. maltaromaticum* or *B. thermosphacta*, in contrast to *S. liquefaciens* growth in the 3-species culture (Supplemental Figure C.7).

Maximum population density

There was no significant difference between *S. liquefaciens* MPD in individual and 3-species culture (Figure 5.11). Unlike *C. maltaromaticum* and *B. thermosphacta*, there was no trend in the difference between MPD for individual and 3-species culture as UDLA increased (Figure 5.12).

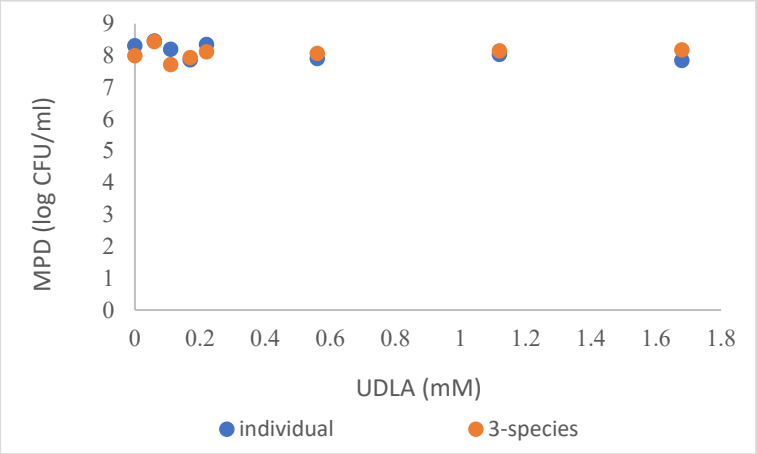


Figure 5.11. *S. liquefaciens* MPD as a function of UDLA in individual versus 3-species culture.

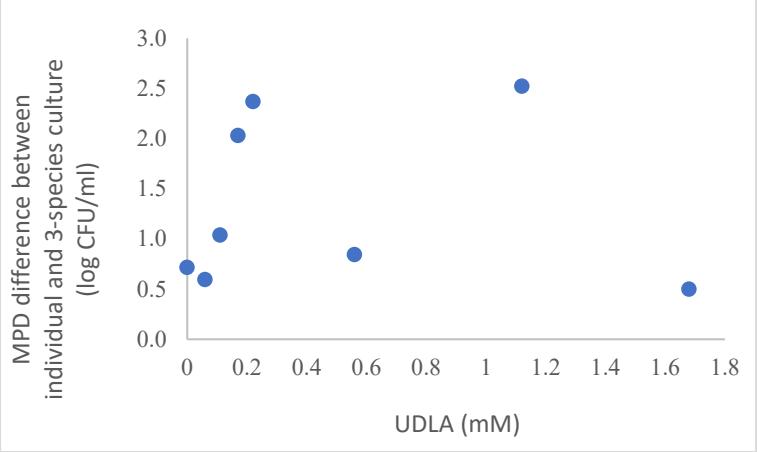


Figure 5.12. Difference in *S. liquefaciens* MPD for individual and 3-species culture as a function of UDLA up to 1.68 mM concentration.

When separately cultured with *C. maltaromaticum* and *B. thermosphacta*, there was little effect (i.e. <0.43 log CFU) on *S. liquefaciens* MPD at any UDLA level (Supplemental Table C.1 and C.2).

5.4.4 Validation of mBHI growth kinetics in VP beef

A separate experiment was performed to validate that growth kinetics in mBHI broth were similar to that of beef. Following irradiation treatment of VP beef, glucose levels, pH, and LA were <5.55 mM, 5.5, and 39 mM, respectively, with a corresponding UDLA level of 0.87 mM. Growth rates for *C. maltaromaticum*, *B. thermosphacta*, and *S. liquefaciens* in beef were 0.049, 0.028, and 0.050, respectively. When beef pH was adjusted to 6.5 (0.09 mM UDLA), growth rates for *C. maltaromaticum*, *B. thermosphacta*, and *S. liquefaciens* were 0.050, 0.065, and 0.056, respectively. Species growth rates in VP beef were then compared to those observed in mBHI broth at 0 mM glucose (see Table 3.2, Chapter 3). Since growth in mBHI had not been measured at 39 mM LA, a linear regression was made between 0 to 50 mM LA, and separately for pH 5.5 and 6.5. B_f and A_f for GR at both 5.5. and 6.5 were 1.11 and 1.12, respectively (Table 5.2), which were acceptable based on the criteria of Oscar (2005).

Table 5.2. Validation of mBHI sqrt GR in VP beef with pure culture of *C. maltaromaticum, B. thermosphacta* and *S. liquefaciens.*

Species	pH	regression formula		observed sqrt GR[2]	predicted sqrt GR[1]
		m	b		
C. maltaromaticum	5.5	-0.0008	0.2464	0.2214	0.2152
	6.5	-0.0002	0.3028	0.2236	0.2950
B. thermosphacta	5.5	-0.0016	0.2304	0.1673	0.1680
	6.5	-0.0009	0.309	0.2550	0.2739
S. liquefaciens	5.5	-0.0005	0.2504	0.2236	0.2309
	6.5	-0.00002	0.3049	0.2366	0.3041

[1] predicted sqrt GR = m * x + b; where x=mM LA

[2] 39 mM LA at pH 5.5 and 6.5 (0.87 and 0.09 mM UDLA), <5.55 mM glucose

5.5 Discussion

Interactions among microbial species within an environment lead to competition for nutrients and space (Lloyd and Allen, 2015), the dynamics of which may be affected by intrinsic (e.g. meat pH, glucose, LA) and extrinsic factors (temperature, package atmosphere) (Buchanan and Bagi, 1997; Newton and Gill, 1978; Shay et al., 1984; Zhang et al., 2017). For example, Buchanan and Bagi (1997) reported that growth suppression of *Listeria monocytogenes* by *Carnobacterium piscicola* was affected by low NaCl, temperature, and pH. Similarly, Zhang et al. (2017) found that inhibition exerted by one *C. maltaromaticum* isolate on another *C. maltaromaticum* isolate

increased with lower pH and higher lactic acid concentration. Microbial interactions may also be affected by inhibitory metabolites produced by microflora (Gram et al., 2002; Russo et al., 2006).

B. thermosphacta, C. maltaromaticum, and *S. liquefaciens* are frequently isolated from refrigerated VP beef. While various reports describe these species during the evolution of beef microbiomes (Casaburi et al., 2011; Ercolini et al., 2006; Gill, 1983; Kaur et al., 2017b), a quantitative description of dynamic interactions among species in a VP beef environment is lacking.

Growth of these three species have been reported for various levels of pH, lactic acid, temperature, glucose, as well as packaging atmospheres in broth, raw or VP meat to achieve different goals (Brightwell et al., 2007; Campbell et al., 1979; Casaburi et al., 2011; Dainty and Hofman, 1983; Grau, 1980), however, these studies did not consider interactions among these three isolates at variations of pH and LA levels.

The experimental design, including heat-shrinking a commercial VP film in a hot water bath at 84˚C for 3 sec was done to more closely resemble commercial VP beef processing (Bell et al., 2001; Narasimha Rao and Sachindra, 2002; Seideman et al., 1976) in which oxygen transmission rate can be reduced from 20 cc/m^2/day to 5 cc/m^2/day (Steve Ward, pers. comm.). Also, utilizing the qPCR method developed in Chapter 4 allowed individual species growth kinetics to be studied for combined effects of pH, LA, and UDLA, thus providing a more mechanistic understanding about how certain bacterial species eventually dominate or are diminished within a beef microbiome.

Insignificant differences in comparison and validation of growth data between the developed qPCR technique (Chapter 4) and plate count method for all three species referred to the justification of adopting molecular technique over convention method to save time, labor and maintain experiment accuracy. Such preference was also was reported by other researchers (Alarcon et al., 2006; Chen et al., 2010; Hein et al., 2001; Hierro et al., 2007; Perelle et al., 2004; Takahashi et al., 2009).

Results showed that *C. maltaromaticum* and *S. liquefaciens* were more resistant to low pH and high UDLA levels compared to *B. thermosphacta*. *B. thermosphacta* GR in the 3-species mixed culture was not affected by increases in UDLA concentrations from 0 to 0.22 mM but decreased significantly at 0.56 and 1.12 mM. In contrast, *B. thermosphacta* MPD was reduced significantly in 3-species mixed culture from 0 to 1.12 mM UDLA. MPD was significantly impacted when grown in a 3-species mixed cultures. This effect was increasing progressively with the increase of UDLA concentration.

Similar findings have been reported for the related species *L. monocytogenes* in mixed culture with *Carnobacterium piscicola* and other background microflora (Buchanan and Bagi, 1997; Carlin et al., 1996), in which *L. monocytogenes* MPD was significantly inhibited. The observation that inhibition of *B. thermosphacta* MPD in the 3-species was caused by *C. maltaromaticum* and *S. liquefaciens*, agrees with Russo et al. (2006) who found *B. thermosphacta* growth was inhibited in the presence of LAB and *Enterobacteriaceae* compared to individual growth on LYP agar. Also, Kaur et al. (2017b), using 16S rRNA amplicon metagenomic data, reported MPD inhibition of *B. thermosphacta* was around 4.4 to 5.4 log CFU/cm^2 stored at 8 and -1.2^{o}C in presence of other meat spoilage organism in VP lamb meat. In that study, *Carnobacterium* and

Serratia were among the dominant taxa and reached populations of 7-8 log CFU/cm^2 while MPD of *B. thermosphacta* was restricted. Significant difference in MPDs was observed at 1.12 mM UDLA (generated from pH 5.5 and LA at 50 mM) in 3- species culture for *B. thermosphacta, C. maltaromaticum* and *S. liquefaciens* (4.26 versus 7.98 and 8.14 log CFU/ml) (Supplemental Table A.2, B.2 and C.2). Newton and Gill (1978) also reported, large populations of *Lactobacillus* (a LAB member) or *Enterobacter* (an *Enterobacteriaceae* member) restricted *B. thermosphacta* growth, possibly by creating glucose limiting conditions on meat surface; however, such factor stimulating inhibition was not investigated in the current study.

The inhibitory activity of LAB is generally mediated by lactic acid produced via glucose fermentation or by the action of bacteriocins (Borch et al., 1996; Mokoena, 2017; Signorini et al., 2006). Collins-Thompson et al. (1983) hypothesized that inhibition of *B. thermosphacta* by *Lactobacillus brevis* in APT broth at pH 4.5 occurred due to excretion of a low molecular weight substance from LAB species which induced autolytic processes in the cell wall of *B. thermosphacta*. The low molecular weight substances could be a type-1 bacteriocin according to Mokoena (2017), which might explain the reduction of *B. thermosphacta* MPD by *C. maltaromaticum* observed in our study. Newton and Gill (1978) found that glucose consumption by *Enterobacter*, the only available carbon source, restricts *B. thermosphacta* growth. This does not explain our observations as glucose was not present in mBHI broth, even though *S. liquefaciens* inhibited *B. thermosphacta* MPD.

B. thermosphacta growth was measured in three test systems (i.e. individual, 2-, and 3-species) using two media formulations (0.22 and 1.12 mM UDLA) to explore the underlying mechanism of MPD inhibition. When cultured individually at 0.22 mM UDLA, *B. thermosphacta* reached

MPD at ~400h, compared to 300, 170, and 130 h in the 3-species culture, and in *C. maltaromaticum-B. thermosphacta* and *S. liquefaciens-B. thermosphacta,* co-cultures, respectively (Supplemental Figure A.4). In the *S. liquefaciens-B. thermosphacta* system, *S. liquefaciens* reached MPD ~90 h before *B. thermosphacta*, compared to ~40 h for *C. maltaromaticum* when cultured with *B. thermosphacta* (Supplemental Figure A.4B and 4C). Similar growth patterns were observed at 0.11 and 0.17 mM UDLA for the individual, 2-, and 3-species; Supplemental Figure A.2 and A.3).

MPD achievement time of *B. thermosphacta* at UDLA 1.12 mM also differed among three test system (Supplemental Figure A.6). In individual growth, *B. thermosphacta* attained MPD at ~400-500h which was fairly similar to the time of MPD achievement in *S. liquefaciens-B. thermosphacta* system (Supplemental Figure A.6C). Pair wise growth of *B. thermosphacta* was not observed in *C. maltaromaticum-B. thermosphacta* system up to 600h (Supplemental Figure A.6B). Along with Gram et al. (2002), several other researchers (Coleman et al., 2003; Delignette-Muller et al., 2006; Gimenez and Dalgaard, 2004; Ross et al., 2000; Stephens et al., 1997) claimed that, MPD suppression of certain species by a dominant one in co-culture system as an obvious “Jameson effect” who reported, high concentration of *Salmonella* Litchfield at stationary phase inhibited the MPD of *Escherichia coli* in mixed culture system (Jameson, 1962). In our study, MPD inhibition of *B. thermosphacta* when grown with *C. maltaromaticum* agrees with observation of Jameson (1962) as MPD of *C. maltaromaticum* at stationary phase strictly suppressed the growth of *B. thermosphacta*. In contrast, MPD of *B. thermosphacta* was also restricted while grown with *S. liquefaciens*, however, a slow growth (GR 0.01 CFU/h) was observed and started several hours later than *S. liquefaciens* achieved MPD. Such observation

indicated that, *S. liquefaciens* did not suppress the growth of *B. thermosphacta* entirely, which is not in agreement with Jameson effect.

According to Shaw and Harding (1984), Borch and Molin (1988) and Egan (1983), LAB, i.e. *Carnobacterium* spp., *Leuconostoc* spp. and *Lactobacillus* spp. dominate in refrigerated vacuum packaged beef due to persistence at low oxygen, resistance to carbon dioxide and tolerance to low pH. *C. maltaromaticum* is a dominant spoilage organism from VP beef that have remarkably long shelf lives (Youssef et al., 2014). Kaur et al. (2017a) also reported the isolation of predominating *Carnobacterium spp.* from unusual long shelf life VP beef primals. The growth study in this current work was conducted by VP of mBHI broth at 99% vacuum and *C. maltaromaticum* growth was not affected in oxygen limiting concentration. GR was not affected in 3-species mixed culture up to 0.22 mM UDLA whereas MPD was reduced significantly at the same UDLA concentration range. In contrast, GR was reduced but MPD was not significantly affected at UDLA < 0.22 mM. An interesting growth occurrence was observed at media 9 containing UDLA 1.68 mM, in which *C. maltaromaticum* grew only in the 3-species mixed culture system but not by itself. Interestingly, a reverse trend of *B. thermosphacta* for MPD inhibition in 3-species mixed culture versus UDLA concentration was observed for *C. maltaromaticum.* From 0 to 0.22 mM UDLA generated from pH 6.5 the MPD difference between individual and 3- species mixed culture decreased gradually with the increase of UDLA concentration. MPD inhibition by 3-species mixed culture was getting even lower at 1.12 and at 1.68 mM UDLA, a strong growth promotion was observed in 3-species mixed culture for *C. maltaromaticum*. No growth was observed for *C. maltaromaticum* when grown with *B. thermosphacta* or *S. liquefaciens* at 1.68mM UDLA. High concentrations of UDLA are either bacteriostatic or bactericidal to several groups of spoilage organisms (Gill, 1986; Greer and Dilts,

1995) and Nassos et al. (1983) reported that, lactic acid concentration increased proportionally with the increase of LAB counts in ground beef stored at 7^{o}C for 18 days. Growth occurrence in higher concentrations of UDLA by *C. maltaromaticum* in presence of other spoilage organism could explain the proliferation and establishment of LAB as a dominant microflora during storage under vacuum condition. Grau (1980) also concluded similar findings.

Neither GR nor MPD of *S. liquefaciens* was affected significantly in 3-species mixed culture where UDLA ranged from 0 to 1.68 mM. Growth was not observed at 2.24 mM UDLA (produced from 100mM LA at pH 5.5) for up to 1200 h. Grau (1981) also reported growth restriction of Gram-negative strains along with *S. liquefaciens* in anaerobic conditions at pH 5.55 buffered nutrient broth containing 100mM L-lactate. In our study, *S. liquefaciens* was detected as the most persistent isolate, showing resistance to vacuum conditions, UDLA concentration up to 1.68 mM as well as bacterial interactions.

Validation study was carried out to investigate the similarity between observed growth data in sterile VP beef and predicted growth data with natural meat of pH 5.5. Negligible variation between observed and predicted GR was observed with meat pH artificially adjusted to 6.5 with NaOH. Though the accuracy factor (A_f) was 1.12, according to Ross (1996) and Oscar (2005), this variation is within the acceptable range for growth predictions from experimental observations.

Initial microbial contamination is unavoidable during slaughter and subsequent processing operations (Kaur et al., 2017b; Stellato et al., 2016) Moreover, environmental factors of storage condition accelerate the proliferation of particular bacterial flora to dominate, leading toward spoilage with specific characteristics. Maintenance of maximum hygiene at abattoirs could be a strategy, in part, to reduce initial contamination. However, scientific interventions are required

to restrict spoilage bacterial growth in beef during storage in vacuum packaged condition at refrigerated temperature.

The apparent growth occurance of *C. maltaromaticum* specifically at 1.68 mM UDLA in 3-species mixed culture mechanistically explained the dominance strategy which could be relevant to naturally contaminated VP beef with a wide range of different microbial groups. However, this observation requires further evaluation in new experiments to define the reliability, the UDLA concentration range it occurs at and the physiological and metabolic mechanistic aspects. Growth restriction of *B. thermosphacta* at pH 5.5- and 50-mM LA or 1.12 UDLA in 3-species mixed culture and the inhibitory effect of *C. maltaromaticum* on *B. thermosphacta* seems to be beneficial for the storage quality of VP beef. Our research findings indicated a concentration of 2.24 mM UDLA (produced from pH 5.5- and 100-mM LA) is inhibitory to these spoilage organisms in individual, 2-species and 3-species mixed culture system. As pH of post rigor muscle ranges between 5.5 to 5.8, beef carcass spraying with 100 mM LA prior to VP might be a possible solution to reduce and restrict spoilage bacterial growth. Additionally, multifactorial growth data considering bacterial interaction could be the basis of multispecies predictive model development of spoilage bacterial growth, a potential practical tool for meat industries to estimate and extend VP beef shelf life.

1

Chapter 6. General Discussion and conclusion

High concentrations of complex nutrients along with the chemical properties of meat supports bacterial growth following initial contamination (Bell and Garout, 1994). Several abiotic factors impose a selection pressure to promote or supress the growth of specific spoilage organisms (Borch et al., 1996). Growth of such organisms to unacceptable levels alters sensorial attributes to the point of customer rejection (Gram et al., 2002). Understanding the effect of growth regulating factors on the succession dynamics of the spoilage microflora during storage is a prerequisite to control their growth. Moreover, an extended shelf-life of beef and beef products is required during international commercial shipment for Australian meat industry. This thesis primarily focuses on the effect of pH, lactic acid and glucose concentration on growth of three potential spoilage organisms (*C. maltaromaticum, B. thermosphacta* and *S. liquefaciens*) under commercial VP condition. A qPCR technique was developed to investigate the growth behaviour of these particular organisms in mixed culture broth system. Finally, the inhibitory effect of undissociated lactic acid (UDLA) was investigated in individual, 2- and 3-species mixed culture broth systems considering bacterial inter-species interactions.

In chapter 3 of this study, levels of pH, glucose and lactic acid (LA) were selected considering beef characteristics to formulate modified BHI (mBHI) broth. A growth study was conducted with commercial VP conditions at an incubation temperature of 10^{o}C using the mBHI broth. Growth data obtained from eight different media with 3 isolates in 2 biological replicates and statistical analysis identified pH as the most effective growth-controlling intrinsic factor. *B. thermosphacta* had the greatest sensitivity to pH with growth rate and MPD. Glucose, at a concentration of 5.55 mM had a minimal effect by comparison. The experimental data

indicated the growth rate of *C. maltaromaticum* was slowed by 40% at pH 5.5 compared to 6.5, which can tolerate pH as low as 3 - 4.5 (Afzal et al., 2013). Several experimental findings justified the low pH tolerance and progressive dominance of *C. maltaromaticum* in VP meat (Kaur et al., 2017a; Zhang et al., 2018), however, Yang et al., (2009) reported maximum growth rate was between pH 6.2 to 7.5. In agreement with our and other studies, Zhang et al., (2018) reported an 18% slower growth rate of *C. maltaromaticum* when pH was reduced from 6.2 to 5.4.

In this study, *B. thermosphacta* showed highest growth inhibition to pH reduction from 6.5 to 5.5 which resulted in two-fold and 1-log reduction in growth rate and MPD, respectively. 70% growth reduction was reported by Papon and Talon (1988) to a pH decrease from 7.0 to 5.6. Additionally, growth study under VP atmospheric conditions might be another explanation behind the manifestation of the strongest effect in growth inhibition. Such inhibition due to anaerobiosis along with a pH decrease was also observed by Campbell et al. (1979). Shifting the pH from 6.5 to 5.5 also adversely affected *S. liquefaciens* with a 40% slower growth rate, which is in agreement with previous report by Gill and Newton (1979). In addition, Grau (1981) included anaerobiosis as another factor that retarded growth of four-gram negative bacteria including *S. liquefaciens.*

LA, a natural constituent of beef (0.9 to 1%) itself, acts as a common bacterial inhibitor both in dissociated and undissociated form due to higher pKa values (Cherrington et al., 1991; Gill, 1986). Such antagonistic effects were also reflected in the growth of the three model strains in this study. Significant reduction in growth rate and MPD was noticed for *C. maltaromaticum* which is supported by other researchers. According to the findings of Jones (2004), a change in LA concentration from 13,700 µg/ml (150 mM) to 17,100 µg/ml (190

mM) altered dominating microflora from C*arnobacterium* spp. to *Leuconostoc mesenteroides* in beef stored at-1.5^{o}C over a period of 16 weeks. Such a shift of dominance occurred due to the relatively weaker tolerance to lactic acid of *Carnobacterium* spp. compared to other LAB strains (Yang et al., 2009). A 54% lower growth rate was reported by Zhang et al. (2018) in the presence of 60 mM LA at pH 5.4 compared to no additional lactic acid at same pH. The inhibitory effects of LA was also observed on the growth of *B. thermosphacta*, which is in agreement with previous finding of Grau (1980). In addition, a complete growth restriction can be achieved at pH 5.5 with 210 mM LA in anaerobic conditions according to Grau (1980). The least significant effect of lactic acid was observed for *S. liquefaciens* in this study, which is consistent with earlier findings of Grau (1981).

Glucose, a primary carbon source for bacterial growth, is metabolised into LA leading to pH reduction which restricts spoilage bacterial growth. Such findings was reported by Shelef (1977) with 2-10% glucose concentration during refrigerated storage of ground beef. In this study, 5.55 mM glucose did not exert a significant impact on the growth rate of the test species except for promoting *B. thermosphacta* MPD., Similar findings were reported by Papon and Talon (1988).

Antibacterial activity (bactericidal or bacteriostatic) and mode of action of undissociated lactic acid is well understood (Greer and Dilts, 1995; Janssen et al., 2007; Podolak et al., 1996; Shelef, 1994). In case of meat the dissociation of LA is dependent on the interaction of pH and LA, thus lower pH produces higher concentration of UDLA, which in turn restricts bacterial growth as its concentration increases (Blixt and Borch, 2002; Signorini et al., 2006). In this study, the highest amount of UDLA (1.12 mM, produced from 50 mM LA at pH 5.5) showed immense effects on growth rate or MPD of all the test species, in particular *B.*

thermosphacta. Research findings by Grau (1980) for *B. thermosphacta* and *S. liquefaciens* inhibition by UDLA was in agreement with the conclusion.

In chapter 4 of this thesis, a 16S rRNA based SYBR-green qPCR technique was developed to observe growth behaviour of specific test species in a mixed culture system. This new method offers several advantages over conventional cultivation-dependent approaches. In this respect, qPCR is a relatively a sensitive technique compared to conventional culture-based method which requires optimisation of each step to avoid incorrect outputs. Temperature gradient PCR for 25 cycles was conducted to select comparatively higher annealing temperature (65^{o}C) for species specific primer selection. Ishii and Fukui (2001), reported a significant effect of annealing temperature and cycle number on discrimination during selective amplification which could be reduced by applying high annealing temperature for community structure studies. Several other researchers recommended to run a low cycle number to limit side product formation, template reannealing biases and most importantly non-specific amplification which was considered in our primer selection step (Qiu et al., 2001; Suzuki and Giovannoni, 1996). Successful reaction specificity of each species was determined by observation of a single peak in the melt curves, initially a minor peak occurred from primer dimer for *S. liquefaciens* which was eliminated by altering the qPCR cycle profile. Such artefacts have been reported previously and represent a limitation of SYBR- green based qPCR. Fortunately, these artefacts were eliminated by selecting a higher melting temperature along with reduced primer concentration as previously shown (Powell et al., 2006). Hence, melt curve analysis is essential to detect non-specific or false positive amplification in method development stage.

According to Svec et al. (2015), a standard curve construction is crucial for absolute quantification of a target gene. qPCR assay performance is usually determined by the efficiency of standard curve which is calculated by the formula considering slope of standard curve, $E= 10^{-1/slope}-1$ (Bustin et al., 2009). The acceptable range of reaction efficiency may vary between 90- 100%, and a value of 1.00 or 100% refers maximum duplication of target gene with the highest efficiency. Reaction efficiency of standard curve was 100% and linear quantification achieved over the range of 5-log concentration for each of the isolate in our study, which is consistent with previous reports (Hein et al., 2005; Martínez-Blanch et al., 2009).

Precision and reproducibility of a developed method is determined by coefficient of variation (CV). Previous studies recommended to consider gene copy number or bacterial concentration over machine generated C_t values due to significant variation between CVs generated from gene copy number and C_t value (Dionisi and Hawkins, 2003; Powell et al., 2006; Schmittgen and Livak, 2008). Such variation was also observed in our study, additionally, a consistent higher CV was observed for inter than intra run, in agreement with the findings by Powell et al. (2006) and Dionisi and Hawkins (2003).

Significant variation was not observed between qPCR and plate count data for the test species GR and MPD in our study. In agreement to our findings, Reichert-Schwillinsky et al. (2009) reported similarities between plate count and qPCR data for *Listeria monocytogenes*. Moreover, predictive model development and evaluation did not indicate any significant variation between the two methods (Guan et al., 2017).

A validation experiment was conducted to check if different concentration of species can be determined in a mixed culture system by qPCR method. A negligible variation (> 0.3 log

CFU/ml) was observed for the three isolates, which led to the conclusion that the qPCR method was able to accurately quantify a single bacterial species within a mixed culture system.

In chapter 5 of this thesis, the developed qPCR method was applied to investigate the effect of UDLA on growth kinetics and interactions of *C. maltaromaticum*, *B. thermosphacta* and *S. liquefaciens* in mixed culture. According to Lloyd and Allen (2015), microbial interaction or competition for nutrients and space is observed among the microbial species living in the same environment. Moreover, such interactions are regulated by several internal and external environmental factors (Buchanan and Bagi, 1997; Newton and Gill, 1978; Shay et al., 1984; Zhang et al., 2017). For example, growth suppression of *Listeria monocytogenes* by *Carnobacterium piscicola* is influenced by NaCl levels, temperature, and pH (Buchanan and Bagi, 1997). A quantitative description of bacterial interaction as a function of pH and LA or UDLA is required to understand the complexity of VP beef spoilage microbiome progression. A study to observe the effect of UDLA on bacterial interaction was conducted using the most frequently isolated species from VP beef considering commercial VP condition and modified BHI (mBHI) broth, relevant to beef composition. The aforementioned validated qPCR method was employed to estimate bacterial growth in mixed culture system to observe the effect of interaction as a function of UDLA. Having qPCR as the preferred method is supported by work from by previous researchers (Alarcon et al., 2006; Chen et al., 2010; Hein et al., 2001; Hierro et al., 2007; Perelle et al., 2004; Takahashi et al., 2009)

As expected, *B. thermosphacta* monocultures showed highest sensitivity to low pH and high UDLA concentration in comparison to *C. maltaromaticum* and *S. liquefaciens*. In mixed culture, *B. thermosphacta* MPD decreased significantly from 0 to 1.12 mM UDLA in the

presence of the two other species. The restricted growth of *B. thermosphacta* in presence of other microbial species has been previously reported. Kaur et al. (2017b) found that, *B. thermosphacta* MPD was restricted to 4.4 to 5.4 log CFU/cm^2 in presence of predominating *Carnobacterium* and *Serratia spp.*, which had population densities of 7-8 log CFU/cm^2 in VP lamb meat. In agreement to our findings, another researcher reported growth restriction of *B. thermosphacta* by the presence of LAB and *Enterobacteriaceae* compared to individual growth on LYP agar medium (Russo et al, 2006). MPD restriction of *L. monocytogenes* (a related species of *B. thermosphacta*) was also reported when co-cultured with *Carnobacterium piscicola* and other background microflora in BHI broth (Buchanan and Bagi, 1997). A possible explanation of glucose limiting conditions on meat surface was observed by Newton and Gill (1978), *B. thermosphacta* growth restricted by *Lactobacillus* (a LAB member) or *Enterobacter* (an *Enterobacteriaceae* member). Such findings could not explain growth restriction of *B. thermosphacta* in our experiment as glucose was not present. The inhibitory effect of LA against several spoilage or pathogenic organisms is well established (Grau et al., 1980; Borch et al., 1996; Mokoena, 2017; Signorini et al., 2006). The hypothesis provided by Collins-Thompson et al. (1983) stated that an induced autolytic process in cell wall of *B. thermosphacta* occurred due to excretion of low molecular weight substance from LAB (*Lactobacillus brevis*). Mokoena (2017), identified those low molecular weight substances as type-1 bacteriocins, such inhibitory compound might be the best explanation of *B. thermosphacta* MPD reduction by *C. maltaromaticum* in this study. Pairwise culture of *B. thermosphacta* with either *C. maltaromaticum* or *S. liquefaciens* was grown along with individual and 3-species culture to observe the underlying mechanism of MPD restriction at 0.22 and 1.12 mM UDLA. Growth curve analysis of individual, 3-species and pairwise culture revealed significant differences in MPD achievement time for *B. thermosphacta* which is

crucial to understand the pattern of inhibitory effect exerted from a dominant species in 2-species culture systems. An inhibited MPD of *Escherichia coli* by high concentration of *Salmonella* Litchfield was reported by Jameson, 1962. Henceforth, growth suppression of a certain species by a dominant one in mixed culture system has been known as the "Jameson effect", which is also acknowledged by several other researchers (Coleman et al., 2003; Delignette-Muller et al., 2006; Gimenez and Dalgaard, 2004; Ross et al., 2000; Stephens et al., 1997). A strict suppression of *B. thermosphacta* MPD by *C. maltaromaticum* at stationary phase in co-culture system agreed with the occurrence of a Jameson effect in this current study. In this respect, *B. thermosphacta* was observed to have a several hours longer lag-time when co-cultured with *S. liquefaciens*. Such an observation is not reflected specifically as part of the Jameson effect.

C. maltaromaticum has been identified as a dominant organism of VP beef with unusually long shelf lives (Kaur et al., 2017a; Youssef et al., 2014) with potential adaptation capability of persistence at low oxygen, resistance to carbon dioxide and tolerance to low pH (Borch and Molin, 1988; Egan, 1983; Shaw and Harding, 1984). In this study, *C. maltaromaticum* GR or MPD showed less sensitivity to bacterial interaction and UDLA concentration except from UDLA 1.68 mM, in which *C. maltaromaticum* grew only in 3-species mixed culture system. MPD inhibition in 3-species culture decreased with the increase of UDLA up to 0.22 mM, which was completely the reverse trend observed for *B. thermosphacta*. A realistic explanation of LAB dominance on VP meat, such as *C. maltaromaticum* in VP beef and lamb when the in presence of background microflora could be attributed to this species tolerance to UDLA. This conclusion is consistent with the results of the mixed culture system examined here.

S. liquefaciens showed the highest resistance to UDLA concentration and bacterial interaction as significant reduction was not observed in GR and MPD in 3-species culture up to 1.68 mM UDLA. In agreement to Grau (1981), growth was not observed at 2.24 mM UDLA in our study. Validation experiment of growth of the individual test species in sterile VP beef was identical with natural meat pH of 5.5, though negligible variation was observed in artificially adjusted meat pH to 6.5 with NaOH. Calculated bias (B_f) and accuracy factor (A_f) were within the range of acceptable value (within 12%) suggested by Ross (1996) and Oscar (2005) concluding growth prediction accuracy.

Experimental findings of this study revealed growth restriction of model species at 2.24 mM UDLA (pH 5.5- and 100 mM LA) in two test systems (individual and 3-species culture). Chilled spraying of 100 mM LA while maintaining meat pH 5.5 prior to VP of beef primals could be an acceptable industrial practice to eliminate spoilage bacterial growth. However, sensorial quality assessment of beef is required prior to large scale application of LA. In addition, administration of *C. maltaromaticum* up to a certain concentration in VP beef can be another strategy to inhibit initially contaminating spoilage organisms such as *B. thermosphacta*. There is no alternative approach other than good hygiene practice in abattoirs to restrict enterobacterial contamination.

In conclusion, this research extends our knowledge regarding influence of intrinsic factors on growth regulation of major VP spoilage organisms signifying beef pH preservation below 5.5 with a LA concentration above 50 mM. Effect of UDLA considering 3-species culture has been reported to observe bacterial interaction with small scale (individual species growth) validation study. Additional validation studies are required to confirm the acceptability of predicted growth data obtained from mBHI broth. In future, media formulation needs to

include more variables or factors i.e. glucose, NaCl to observe effects on bacterial interaction. In addition, VP beef spoilage species from different genus need to be incorporated in mixed culture system to understand the complexity of natural microbiome. In future, a multispecies predictive model considering bacterial interaction along with multiple factors would be an essential tool for food industries to understand microbial behaviour and control spoilage in food matrix to ensure safety and quality of ultimate product.

Appendix A

Objective

To select target and effector isolated for thesis studies.

Materials and Methods

Bacterial isolates

Seventeen isolates representing three genera (*Carnobacterium, Serratia, and Brochothrix*) were evaluated in this study. Isolate code and species are listed below.

Serratia spp.	E8c, E30j, E30g, E30h, C0b, C30b, E8i, B0h, D0d, D0c
Brochothrix thermosphacta	A0b, A8f
Carnobacterium spp.	D8c, C30h, C0a, D0h, B0f

Each of the isolates were evaluated as target and effector to detect interactive behaviour. Combinations for each interaction are given below (Table 1).

Table 1. Combinations of target effector isolates

Target	Effector	Target	Effector	Target	Effector
A0b	E8c	E30j	A0b	C0b	C0a
	E30j		A8f		C30h
	E30g		D8c		D0h
A0b	E30h	E30j	C0a	C0b	C8h
	C0b		C30h		B0f
	C30b		D0h	C30b	A0b
A0b	E8i	E30j	C8h		A8f

	B0h		B0f		D8c
	D0c	E30g	A0b	C30b	C0a
A0b	D0d		A8f		C30h
A8f	E8c		D8c		D0h
	E30j	E30g	C0a	C30b	C8h
	E30g		C30h		B0f
A8f	E30h		D0h	E8i	A0b
	C0b	E30g	C8h		A8f
	C30b		B0f		D8c
A8f	E8i	E30h	A0b	E8i	C0a
	B0h		A8f		C30h
	D0d		D8c		D0h
A8f	D0c	E30h	C0a	E8i	C8h
E8c	A0b		C30h		B0f
	A8f		Doh	B0h	A0b
	D8c	E30h	C8h		A8f
E8c	C0a		B0f		D8c
	C30h	C0b	A0b	B0h	C0a
	D0h		A8f		C30h
E8c	C8h		D8c		D0h
	B0f				

Target	Effector	Target	Effector
B0h	C8h	C30h	C0b
	B0f		C30b
D0d	A0b		E8i
	A8f	C30h	B0h
	D8c		D0d
D0d	C0a		D0c
	C30h	C0a	A0b
	D0h		A8f
D0d	C8h		E8c
	B0f	C0a	E30j
D0c	A0b		E30g
	A8f		E30h
	D8c	C0a	C0b
D0c	C0a		C30b
	C30h		E8i
	D0h	C0a	B0h
D0c	C8h		D0d
	B0f		D0c
A0b	D8c	D0h	A0b
	C30h		A8f
	C8h		E8c
A0b	C0a	D0h	E30j
	Doh		E30g
	B0f		E30h

A8f	D8c	D0h	C0b
	C30h		C30b
	C8h		E8i
A8f	C0a	D0h	B0h
	D0h		D0d
	B0f		D0c
D8c	A0b	B0f	A0b
	A8f		A8f
	E8c		E8c
D8c	E30j	B0f	E30j
	E30g		E30g
	E30h		E30h
D8c	B0h	B0f	C0b
	D0d		C30b
	D0c		E8i
D8c	C0b	B0f	B0h
	C30b		D0d
	E8i		D0c
C30h	A0b		
	A8f		
	E8c		
C30h	E30j		
	E30g		
	E30h		

Primary screening

18 target and effector isolates were streaked on TSA plates from a -80°C freezer, incubated at 25°C for 48 h, and then cultured in BHI broth at 25°C for 24 h. 100 µl of overnight cultures was spread-plated on TSA and then three 10 µl aliquots of the effector isolate spotted to the agar surface. TSA plates were incubated at 25°C for 72 h in anaerobic conditions (< 1.0% O2, ≥13% CO2), created by a GasPak EZ anaerobic pouch (BD, Australia) placed in a sealed jar. The inhibition zones were photographed after 72 h.

Secondary screening

Based on the results described in the preceding paragraph, 12 isolates were selected for further study. Two target (A0b and A8f) and 10 effector (B0f, C0a, C0b, C30h, D0c, D0d, D0h, D8c, E30j and E30g) isolates were cultured as described above. 100 µl of each overnight culture was spread-plated on TSA plates and then a single 10 µl aliquot of effector isolate spotted on the agar surface. After drying the agar surface, TSA plates were incubated at 25°C for 72 h in an anaerobic jar, and then inhibition zones photographed.

Quantitative screening

Following evaluation of the 12 isolates described in the preceding paragraph, six isolates were selected based on species and size of inhibition zone. Two target (A0b and A8f) and four effector (B0f, C0a, D0c, D0d) isolates were evaluated using three biological replicates. After incubating isolates at 25°C for 48 h in BHI broth, cultures were streaked on TSA and incubated at 25°C for 24 h. Broth optical density (OD) of effector and target isolates was adjusted to 0.6 - 0.7 and 0.1 - 0.2 respectively, at 600nM wavelength. Then, 100 µl of the target culture spread-plated on TSA, onto which 10 µl of effector isolate was spotted. Once effector droplets dried on the plate, TSA was incubated at 25°C for five days in an anaerobic jar. Inhibition zones

were photographed and then the zone diameter measured using Image J software (version 1.49; [http://rsb.info.nih.gov/ij/index.html]).

Results

Primary screening

There were 15 interactions of inhibition in eight plates comprising target A0b, A8f and effector strains were C0a, D0c, D0d, D0h, B0f, D8c, C30h, C0b, E30j and E30g after 72 h of anaerobic incubation (Figure 1-8).

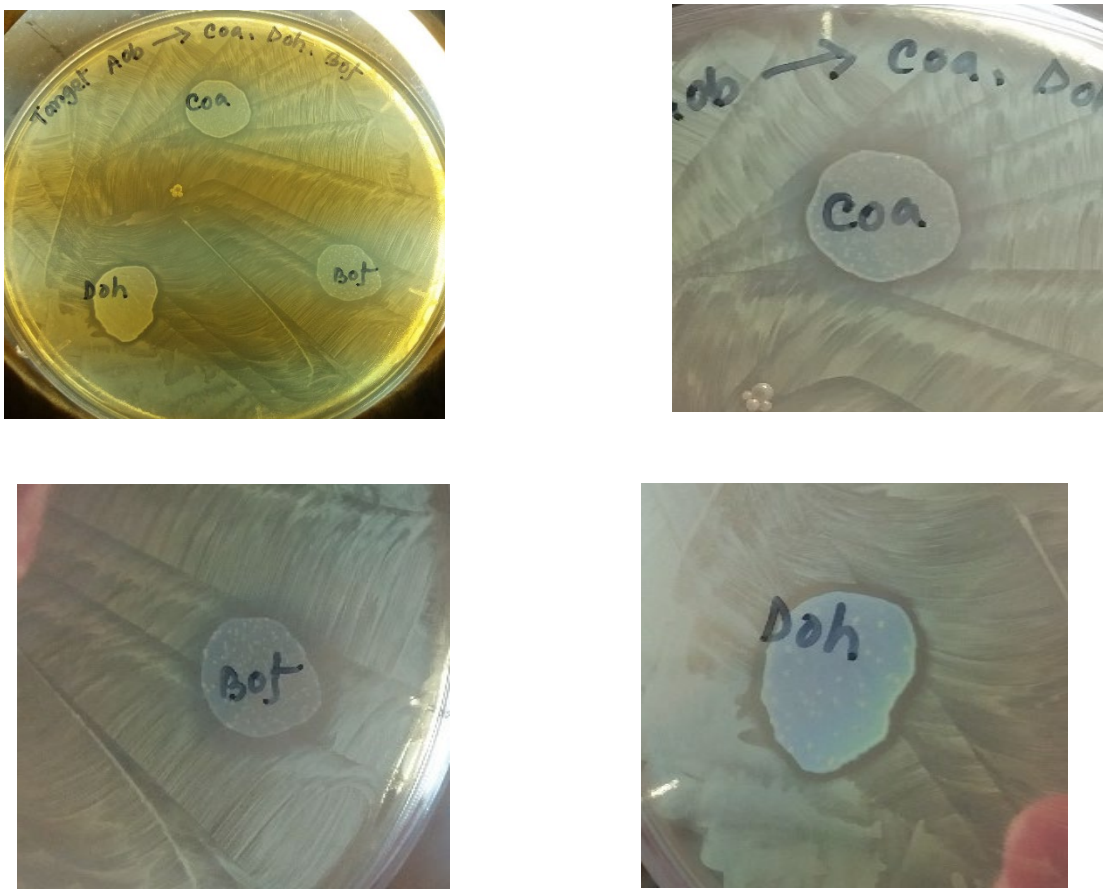


Figure 1. Inhibition of target A0b by effector C0a, B0f and D0h

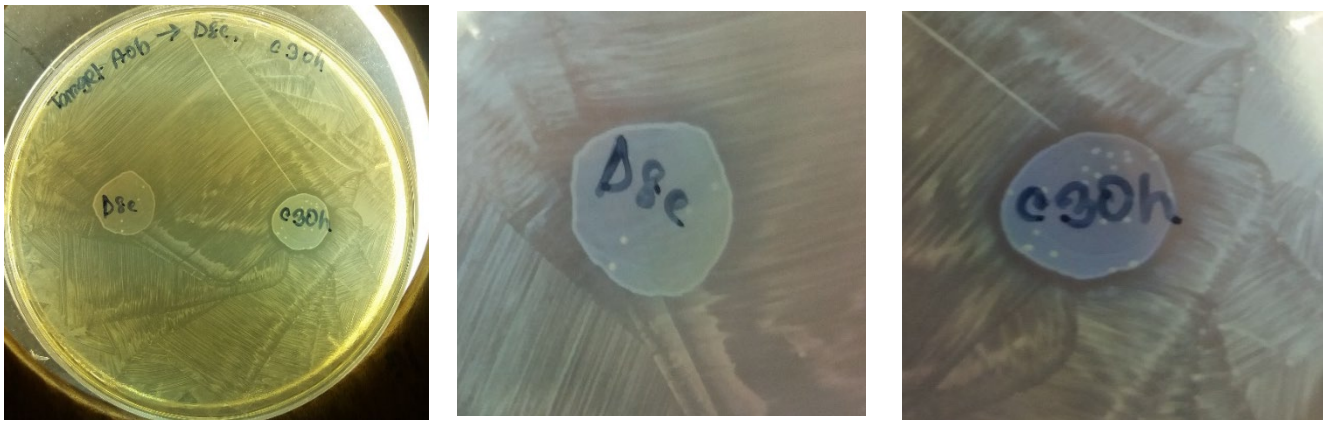


Figure 2. Inhibition of target A0b by effector C30h and D8c

Figure 3. Inhibition of target A0b by effector C0b

Figure 4. Inhibition of target A8f by effector C0a, B0f, D0h

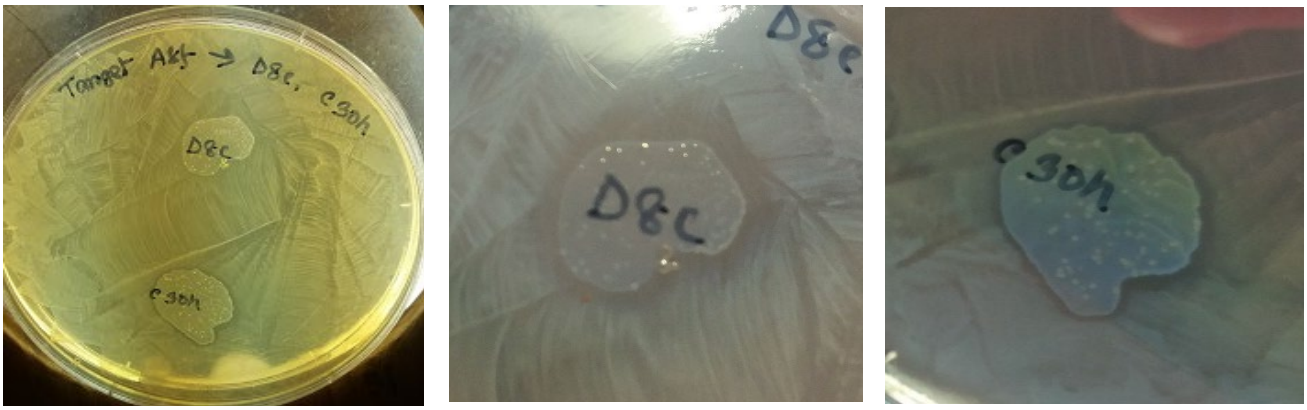


Figure 5. Inhibition of target A8f by effector D8c, C30h

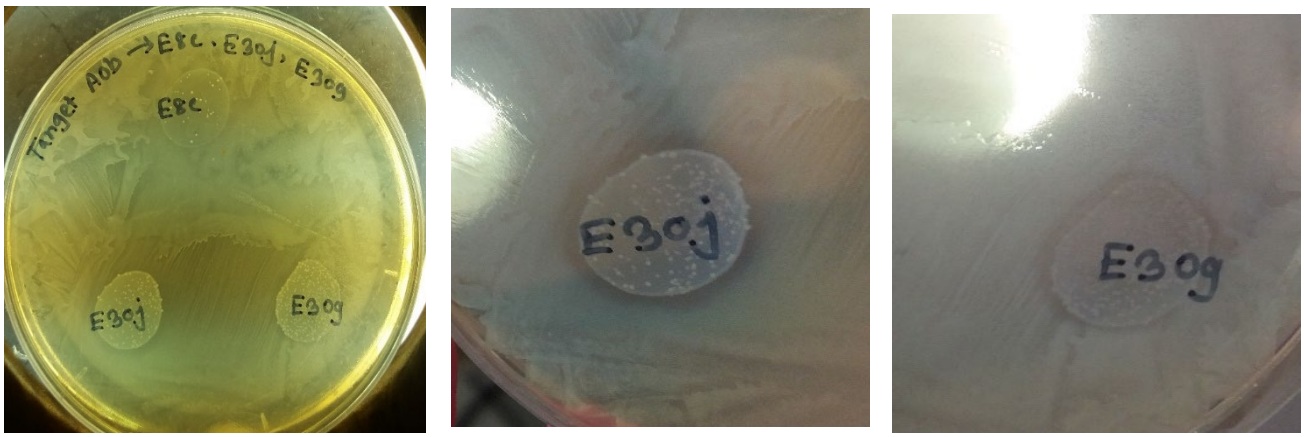


Figure 6. Inhibition of target A0b by effector E30j, E30g

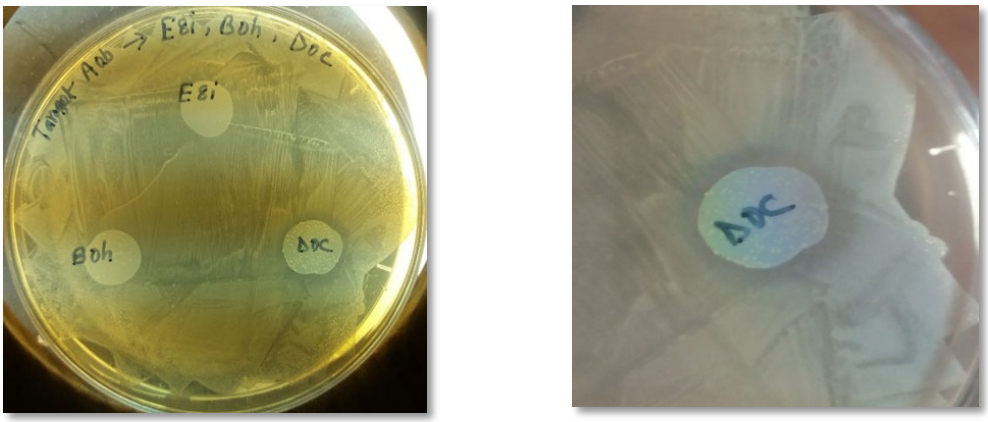


Figure 7. Inhibition of target A0b by effector D0c

Figure 8. Inhibition of target A0b by effector D0d

Secondary screening

12 interactions of inhibition in 12 plates comprising target A0b, A8f and effector strains were C0a, D0d, D0h, B0f, D8c, C30h after 72 h of anaerobic incubation (Figure 9-20, Table 2). 4 interactions out of 15 were not observed in secondary screening (Figure 21-24, Table 2).

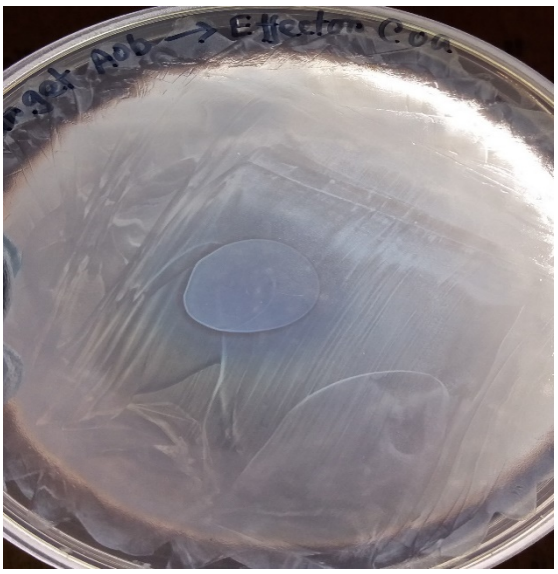


Figure 9. Inhibition of target A0b by effector C0a

Figure 10. Inhibition of target A0b by effector B0f

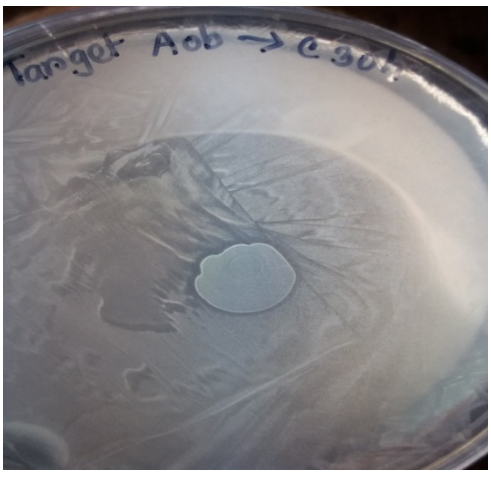


Figure 11. Inhibition of target A0b by effector C30h

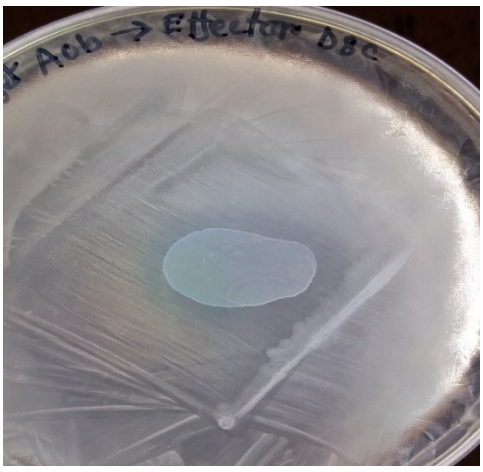


Figure 12. Inhibition of target A0b by effector D8c

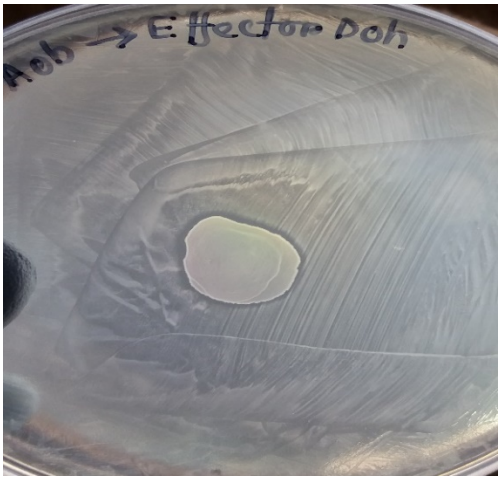


Figure 13. Inhibition of target A0b by effector D0h

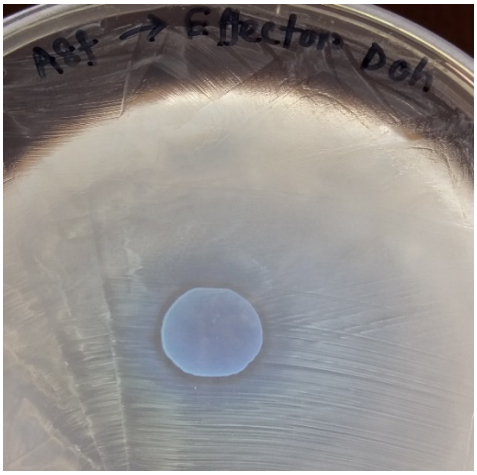


Figure 14. Inhibition of target A8f by effector D0h

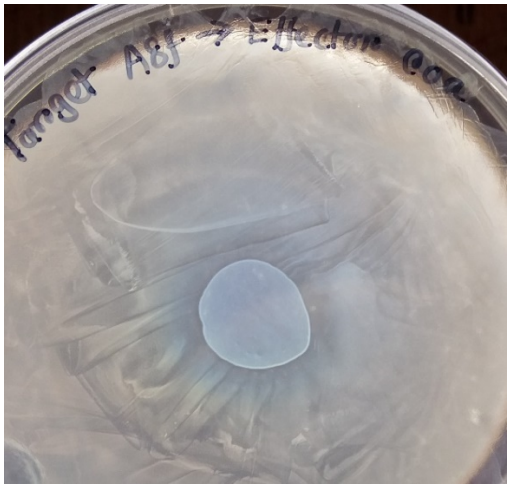


Figure 15. Inhibition of target A8f by effector C0a

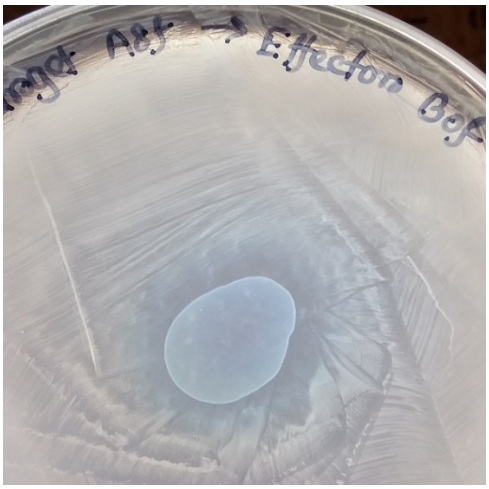


Figure 16. Inhibition of target A8f by effector B0f

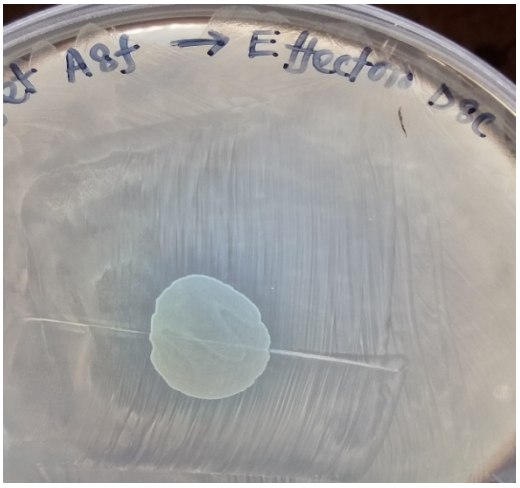


Figure 17. Inhibition of target A8f by effector D8c

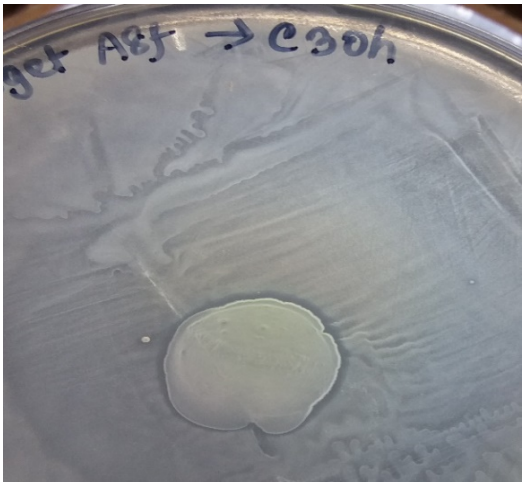


Figure 18. Inhibition of target A8f by effector C30h

Figure 19. Inhibition of target A8f by effector D0d

Figure 20. Inhibition of target A0b by effector D0d

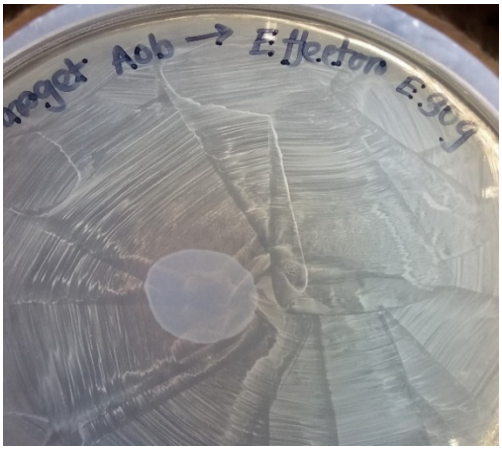


Figure 21. No interaction between target A0b and effector E30g

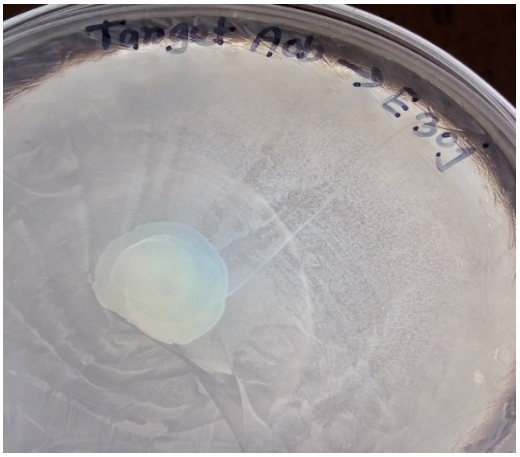


Figure 22. No interaction between target A0b and effector E30j

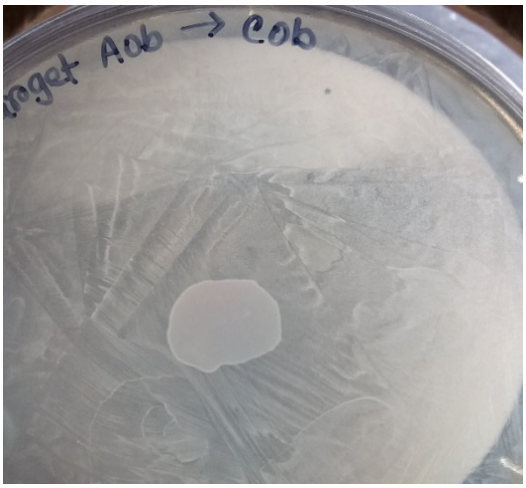


Figure 23. No interaction between target A0b and effector C0b

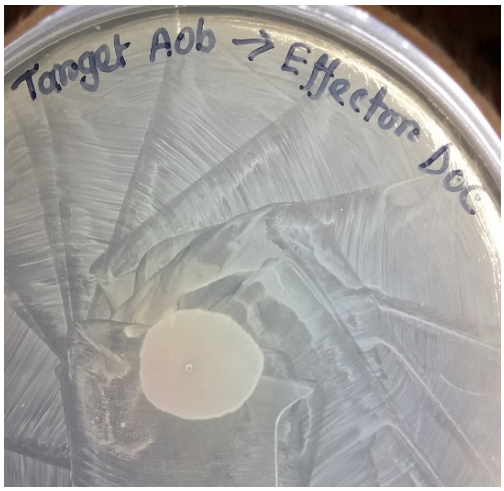


Figure 24. No interaction between target A0b and effector D0c

Each of these isolates were used as a target and effector to screen interactive behaviour with each other. The combination for each interaction and repetition is given in the table below.

Table 2. Summary of primary and secondary screening

Target	**Effector**	**Results**	
		Interaction (1st experiment)	**Interaction (2nd experiment)**
A0b	C0a	Yes	Yes
	B0f	Yes	Yes
	D0h	Yes	Yes
A0b	C30h	Yes	Yes
	D8c	Yes	Yes
A0b	C0b	Yes	No
A8f	C0a	Yes	Yes
	D0h	Yes	Yes
	B0f	Yes	Yes
A8f	D8c	Yes	Yes
	C30h	Yes	Yes
A0b	E30j	Yes	No
	E30g	Yes	No
A0b	D0c	Yes	No
A0b	D0d	Yes	Yes
A8f	D0d	No	Yes

Quantitative screening

The seven interactions comprising two target and four effector isolates were positively found in all biological replicates in third experiment with specific OD (600_{nm}). Based on the diameter of inhibition zone and standard deviation of replicates, the inhibition of target A8f by C0a and D0d were found potential.

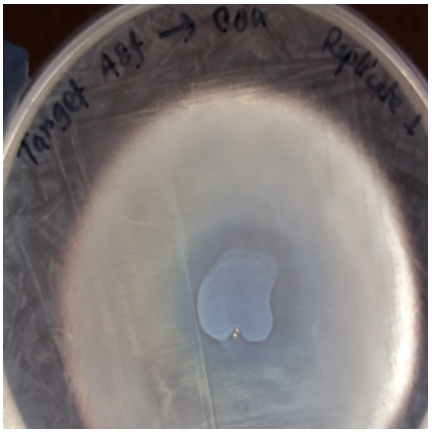


Figure 25. Inhibition of target A8f by effector C0a (replication 01)

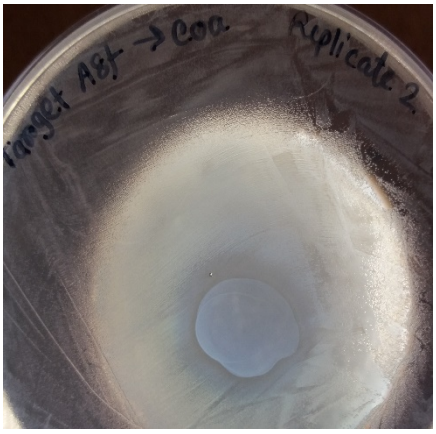


Figure 26. Inhibition of target A8f by effector C0a (replication 02)

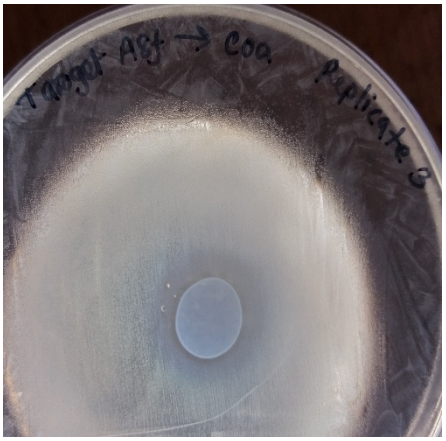


Figure 27. Inhibition of target A8f by effector C0a (replication 03)

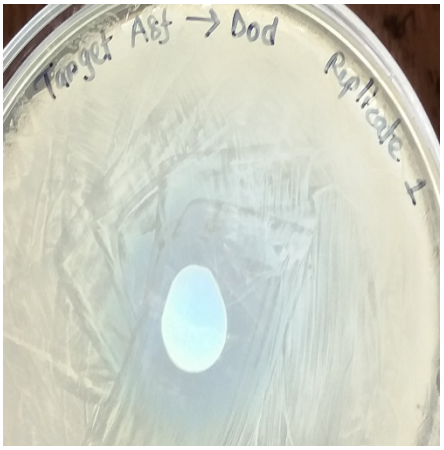


Figure 28. Inhibition of target A8f by effector D0d (replication 01)

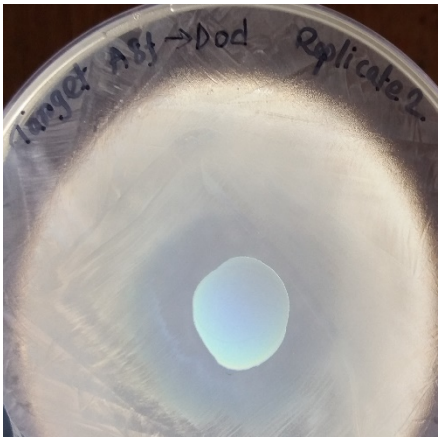


Figure 29. Inhibition of target A8f by effector D0d (replication 02)

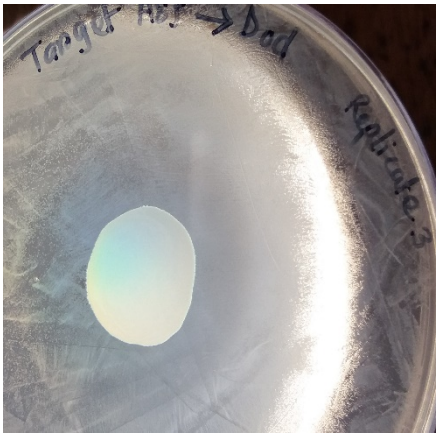


Figure 30. Inhibition of target A8f by effector D0d (replication 03)

Table 3. Summary of quantitative screening

Plate no.	Interaction		Replication	Diameter of inhibition zone (mm)	Average diameter of each replication (mm)	Average diameter of all replications (mm)	Standard deviation of each replication (mm)
	Target	Effector					
1	A0b (B.t)	B0f (C.m)	R1	2.47	2.16	2.43	0.22
				1.86			
				2.14			
2			R2	2.49	2.70		
				2.66			
				2.96			
3			R3	3.07	2.44		
				2.6			
				1.65			
4	A0b (B.t)	C0a (C.m)	R1	3.39	3.29	3.13	0.19
				3.73			
				2.74			
5			R2	3.07	3.25		
				2.75			
				3.92			
6			R3	2.67	2.86		
				2.72			
				3.18			
7	A0b (B.t)	D0d (S.l)	R1	3.44	3.52	3.64	0.52
				3.76			
				3.37			
8			R2	2.58	3.08		
				3.88			
				2.78			
9			R3	4.62	4.33		
				3.86			
				4.5			
10	A8f (B.t)	B0f (C.m)	R1	2.8	2.95	3.05	0.13
				3.14			
				2.9			
11			R2	3.17	2.97		
				2.57			
				3.16			
12			R3	3.69	3.23		
				3.12			
				2.88			

Plate no.	Interaction		Replication	Diameter of Inhibition zone (mm)	Average diameter of each replication (mm)	Average diameter of all Replication (mm)	Standard deviation of each replication (mm)
	Target	Effector					
13	A8f (B.t)	C0a (C.m)	R1	5.09	5.01	4.92	0.11
				4.99			
				4.95			
14			R2	4.61	4.77		
				4.8			
				4.9			
15			R3	4.88	4.99		
				4.98			
				5.1			
16	A8f (B.t)	D0c (S.l)	R1	6.32	5.05	6.69	1.37
				4.12			
				4.72			
17			R2	6.48	6.62		
				6.28			
				7.09			
18			R3	8.04	8.40		
				8.4			
				8.77			
19	A8f (B.t)	D0d (S.l)	R1	7.93	8.17	8.13	0.04
				8.38			
				8.2			
20			R2	7.75	8.08		
				8.77			
				7.71			
21			R3	7.1	8.13		
				8.44			
				8.85			

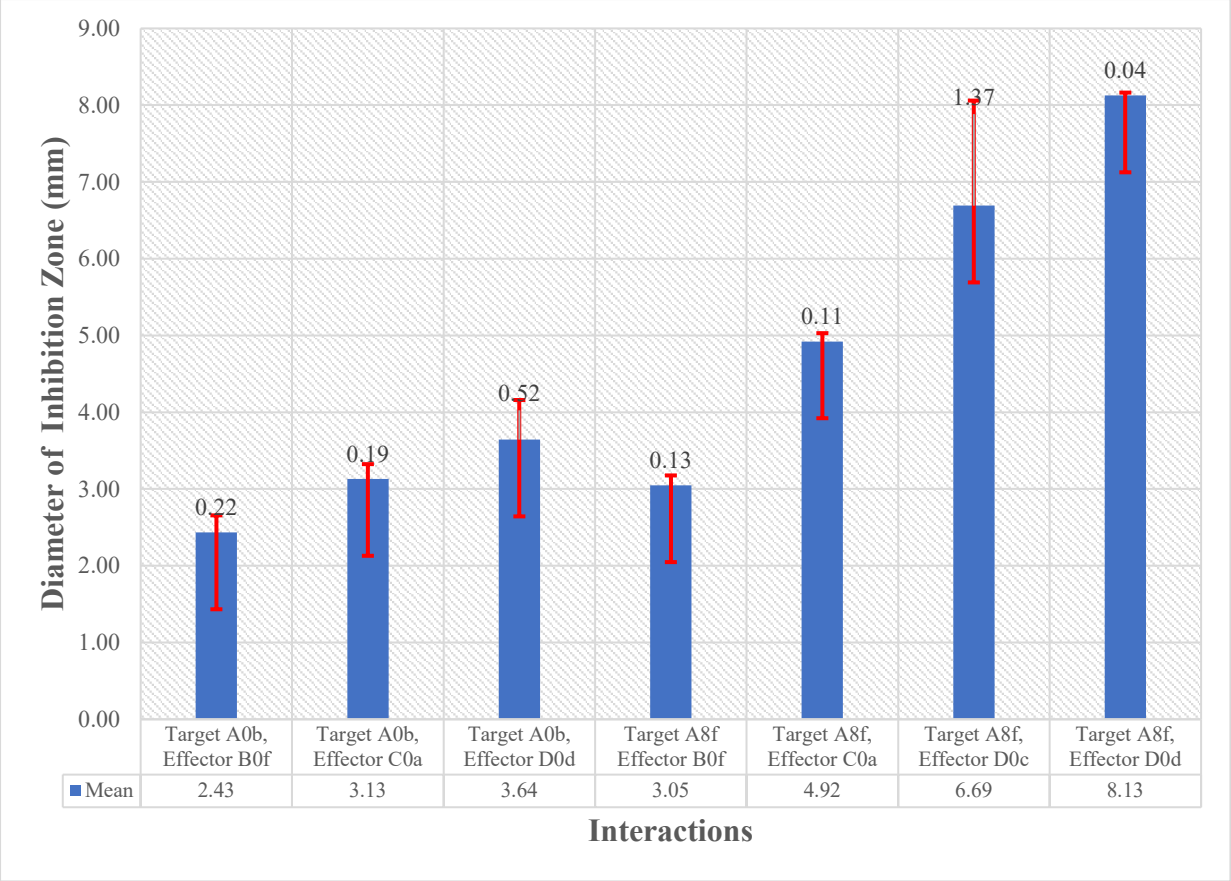


Figure 31. Diameter of inhibition zones of selected interactions and standard deviations

Discussion

Primary screening

From the photographs of interactions above and previous data, it is found that, *Brochothrix thermosphacta* (A0b, A8f) is inhibited by *Carnobacterium* spp. (D8c, C30h, C0a, D0h, B0f) and *Serratia* spp. (E30g, E30j, D0d, D0c). However, the interaction level between target A0b and effector D0h, D8c and C30h can be ignored as the inhibition zones were very weak. Likewise, the interaction levels between target A8f and effector D0h and C30h can be ignored as it is also not strong enough. On the contrary, inhibition of target A0b and A8f is strongly performed by C0a and B0f.

In addition, the inhibition level between target A0b and effector C0b, E30g is very weak and can be ignored whereas, interaction between A0b and E30j can be considered as a significant one. From previous data it was also found that, target A0b was strongly inhibited by effector D0d and D0c. No such interaction was found between target A8f and effector D0c, D0d, C0b, E30g, E30j.

Secondary screening

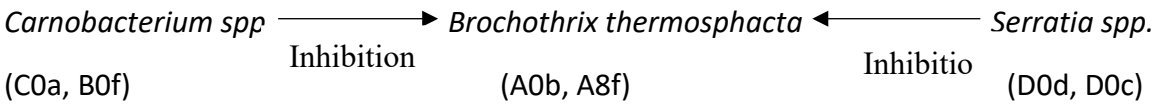
In secondary screening, 4 interactions out of 15 were not observed (target A0b and effector C0b, target A0b and effector D0c, target A0b and effector E30j, target A0b and E30g). Those combinations of target and effector isolates were ignored previously due to very weak interaction level. Though, a new interaction was observed between target A8f and effector D0d which confirmed again that *Serratia liquifaciens* inhibited *Brochothrix thermosphacta* like target A0b and effector D0d. Significantly, the diameter of inhibition zone

between target A8f, effector D0d was much bigger than the diameter of zone of inhibition between A0b and D0d. Interaction between target A0b, effector C0a and target A8f, effector C0a were similar like first experiment. Likewise, interactions between target A0b, effector B0f and target A8f, effector B0f were also same as previous.

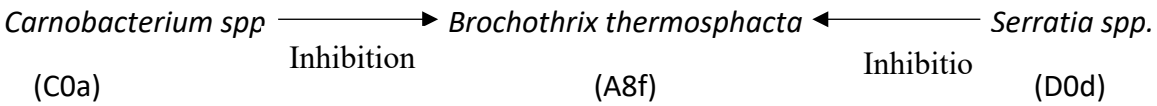
Quantitative screening

In final quantitative screening, the interactions that were repeated in second experiment were validated with replicates by adjusting the optical density of target and effector isolates. By analysing the diameter of inhibition zone and standard deviation, target A8f (*Brochothrix thermosphacta*) was strongly inhibited by both effector C0a (*Carnobacterium maltaromaticum*) and D0d (*Serratia liquifaciens*).

From the first experiment the isolates can be chosen by considering the following figure:



From the second and third experiment the isolates can be confirmed to:

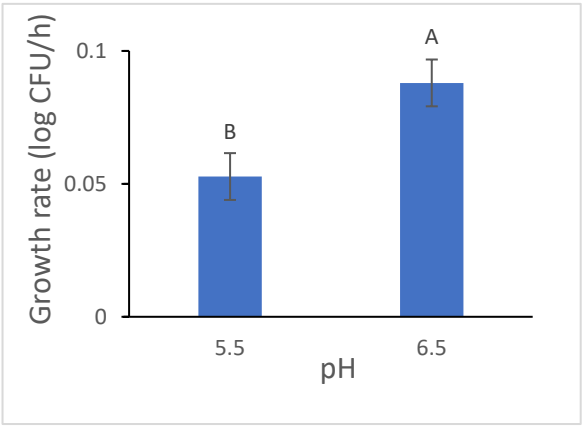


Conclusion

Finally, from the third experiment it can be concluded that, we can consider A8f, C0a and D0d as our desired isolate for further study as these isolates have a linear interaction among themselves and three of the isolates are from three different genera. Moreover, all of them are considered as highly potential spoilage organisms of vacuum packaged beef. Designing of qPCR with the three isolates will be very effective to define specific spoilage criteria of vacuum packaged beef.

Supplemental Figures of Chapter 3

A



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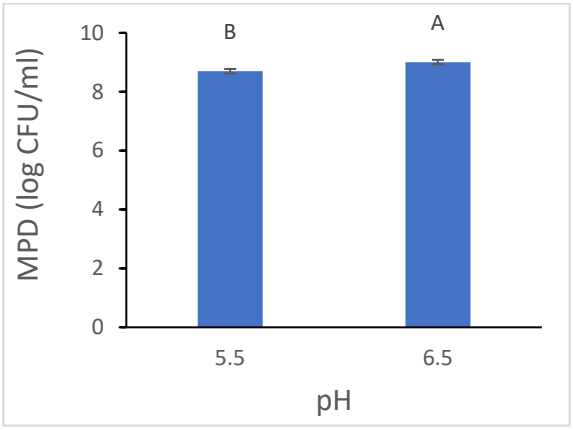

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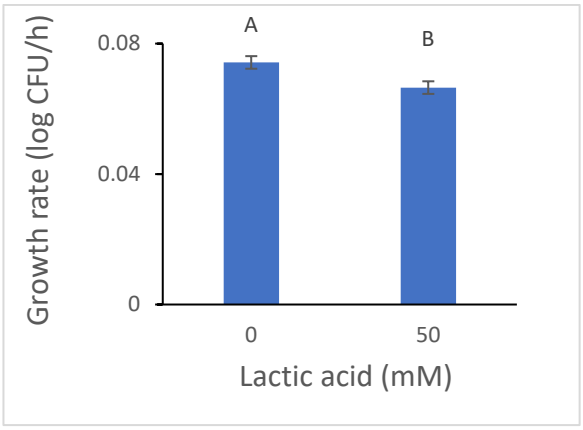

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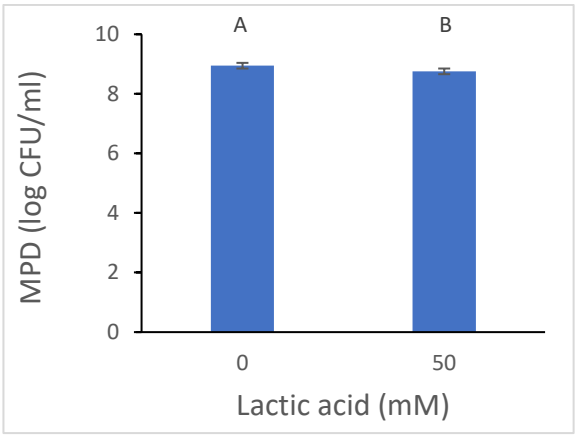

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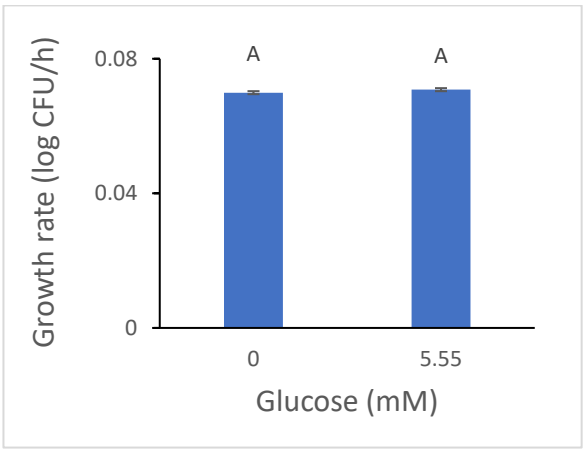
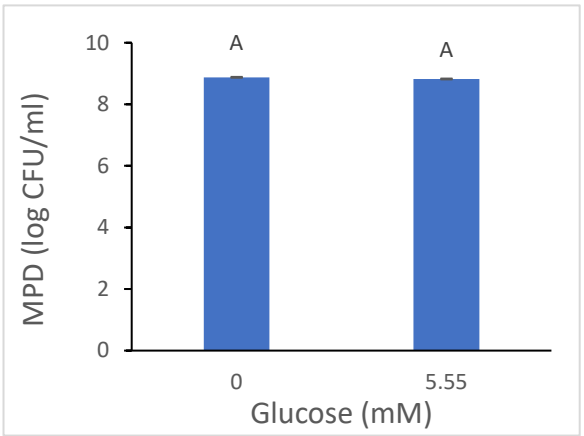


F



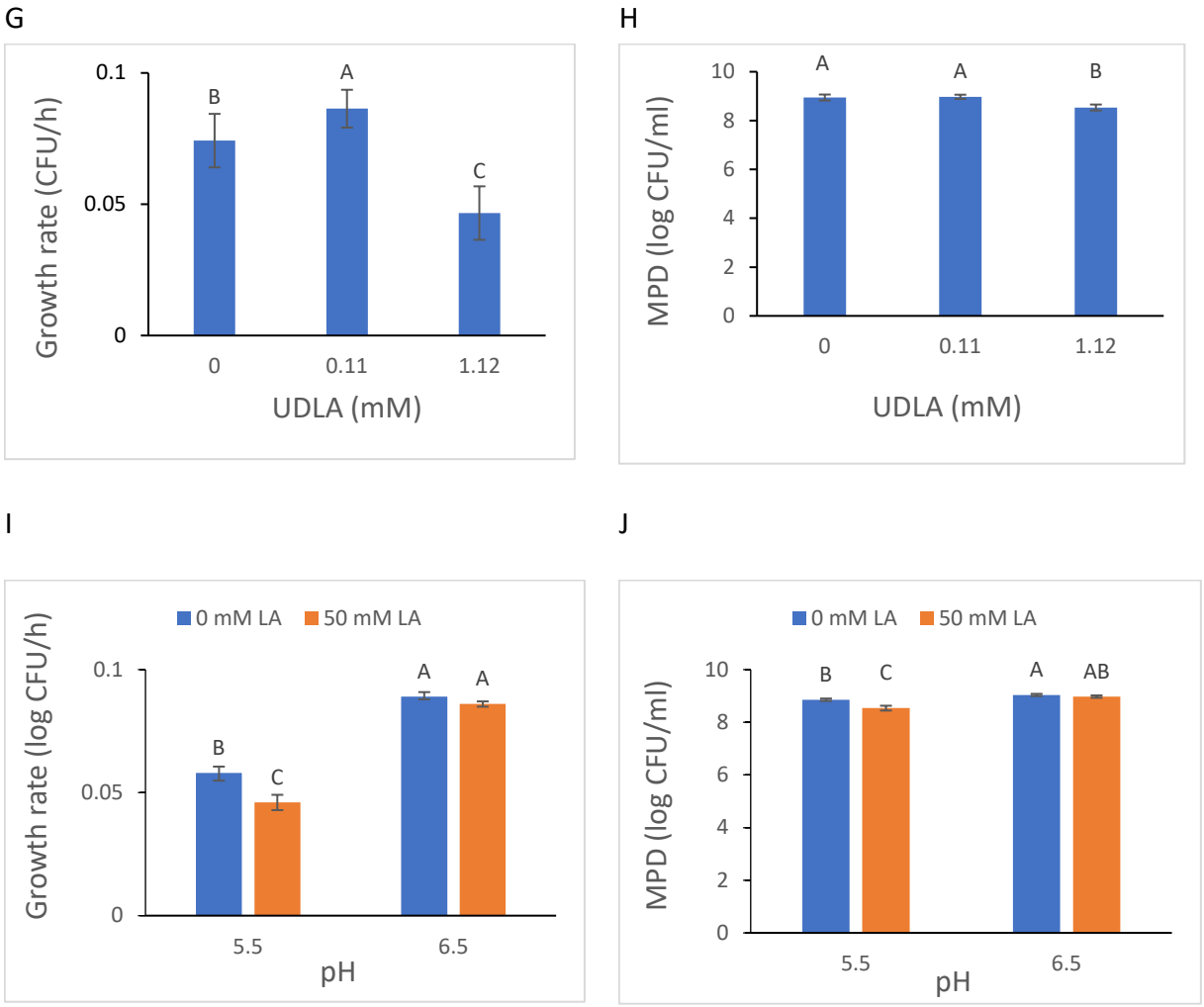
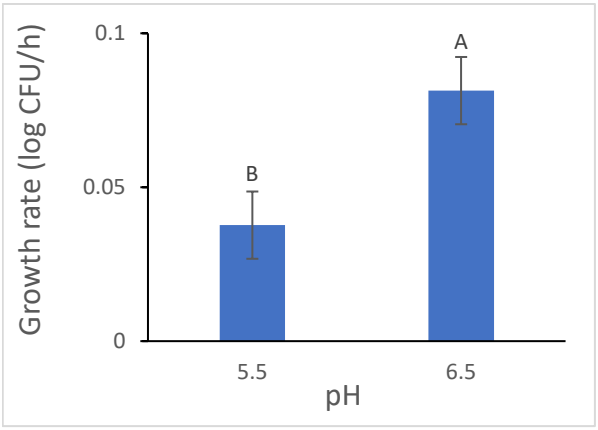
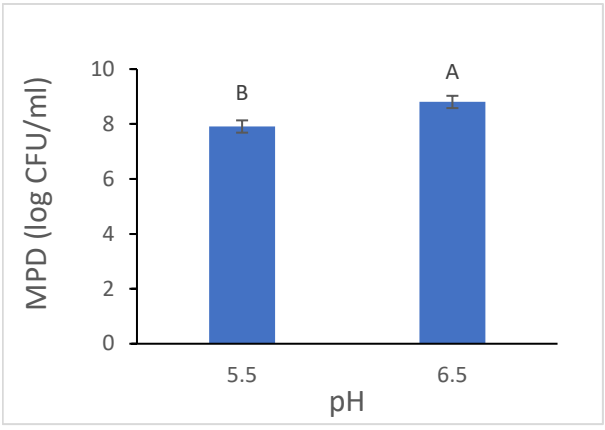


Figure 2. Effect of pH (A, B), lactic acid (C, D), glucose (E, F), UDLA (G, H) and the interaction of pH and lactic acid (I, J) on the GR and MPD of *C. maltaromaticum*. Error bars represent the standard error of the mean. Means with the same letter in each panel were not significantly different.

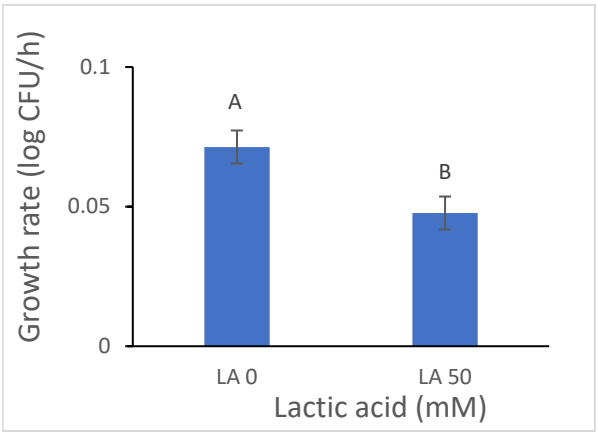
A


Growth rate (log CFU/h)
0.1
0.05
0
B
A
5.5
6.5
pH

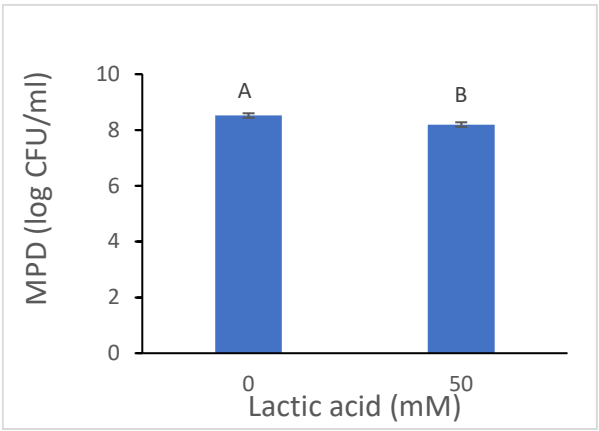
B


MPD (log CFU/ml)
10
8
6
4
2
0
B
A
5.5
6.5
pH

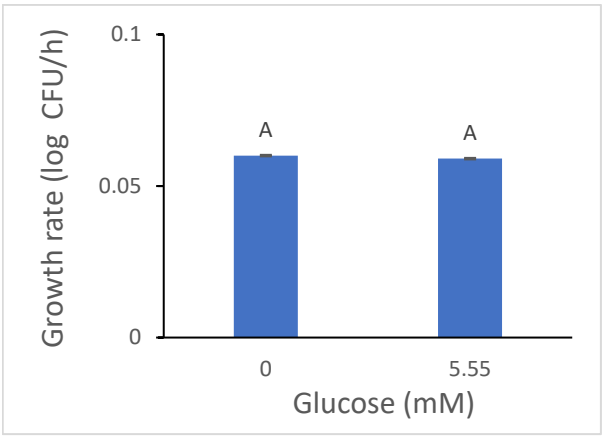
C


Growth rate (log CFU/h)
0.1
0.05
0
A
B
LA 0
LA 50
Lactic acid (mM)

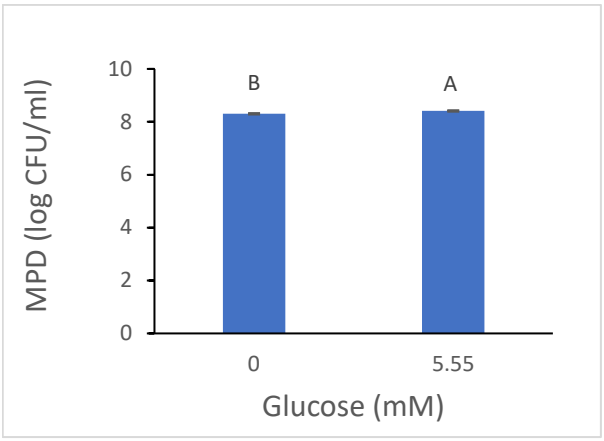
D


MPD (log CFU/ml)
10
8
6
4
2
0
A
B
0
50
Lactic acid (mM)

E


Growth rate (log CFU/h)
0.1
0.05
0
A
A
0
5.55
Glucose (mM)

F


MPD (log CFU/ml)
10
8
6
4
2
0
B
A
0
5.55
Glucose (mM)

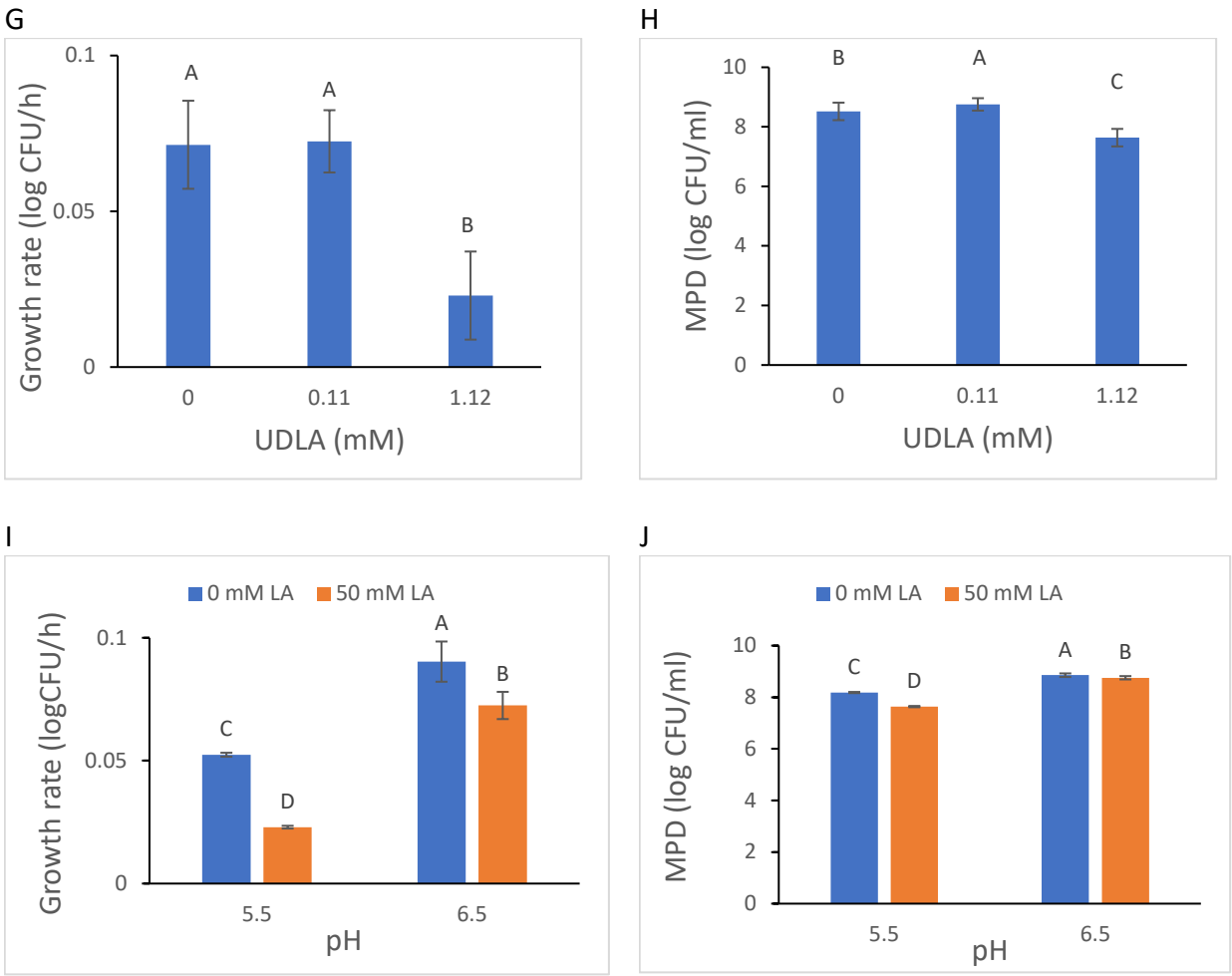
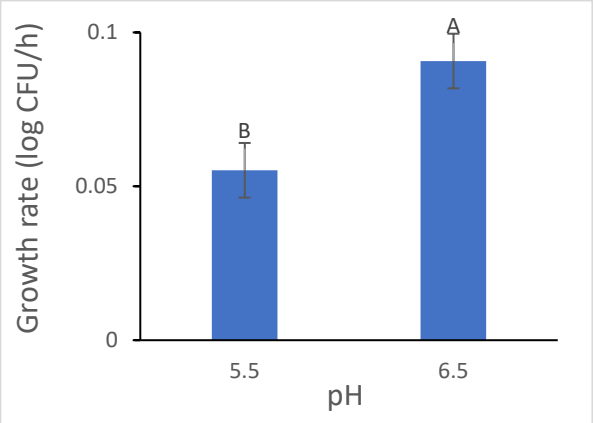
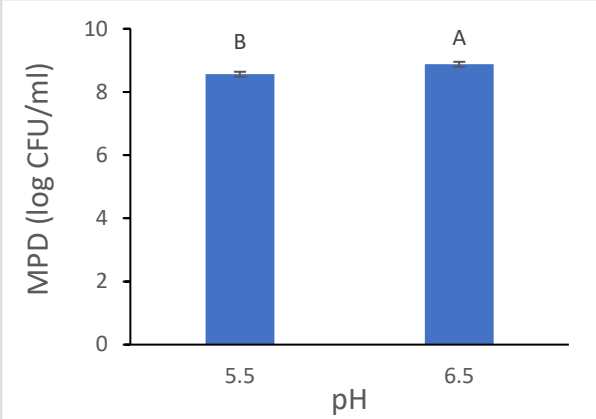


Figure 3. Effect of pH (A, B), lactic acid (C, D), glucose (E, F), UDLA (G, H) and the interaction between pH and lactic acid (I, J) on the GR and MPD of *B. thermosphacta*. Error bars represent the standard error of the mean. Means with the same letter in each panel were not significantly different.

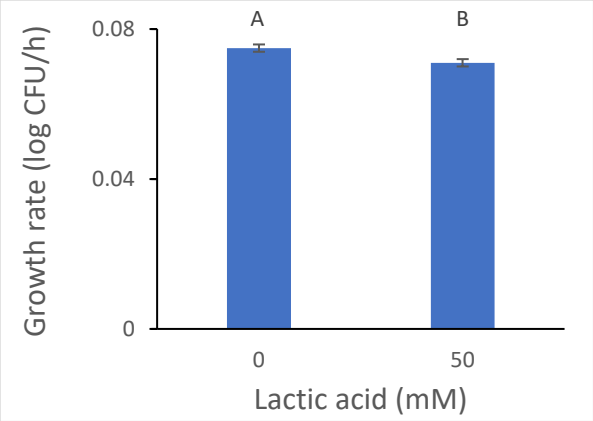
A


Growth rate (log CFU/h)
0.1
0.05
0
B
A
5.5
6.5
pH

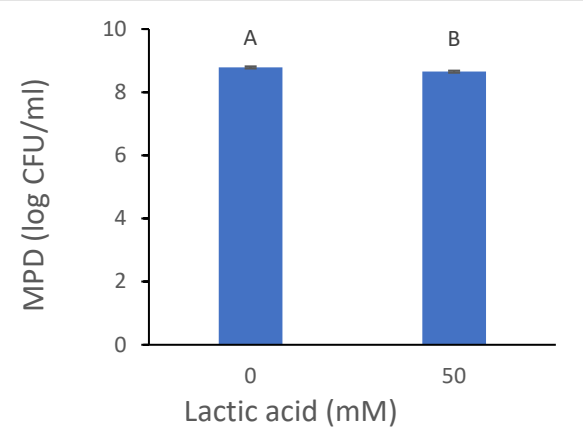
B


MPD (log CFU/ml)
10
8
6
4
2
0
B
A
5.5
6.5
pH

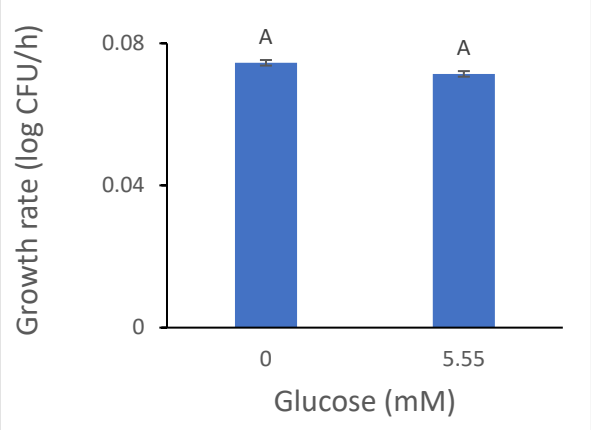
C


Growth rate (log CFU/h)
0.08
0.04
0
A
B
0
50
Lactic acid (mM)

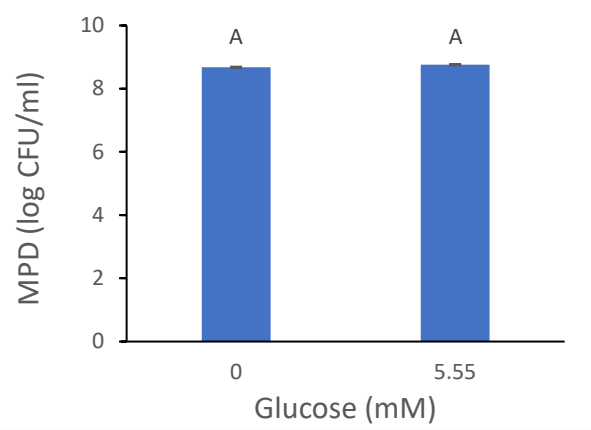
D


MPD (log CFU/ml)
10
8
6
4
2
0
A
B
0
50
Lactic acid (mM)

E


Growth rate (log CFU/h)
0.08
0.04
0
A
A
0
5.55
Glucose (mM)

F


MPD (log CFU/ml)
10
8
6
4
2
0
A
A
0
5.55
Glucose (mM)

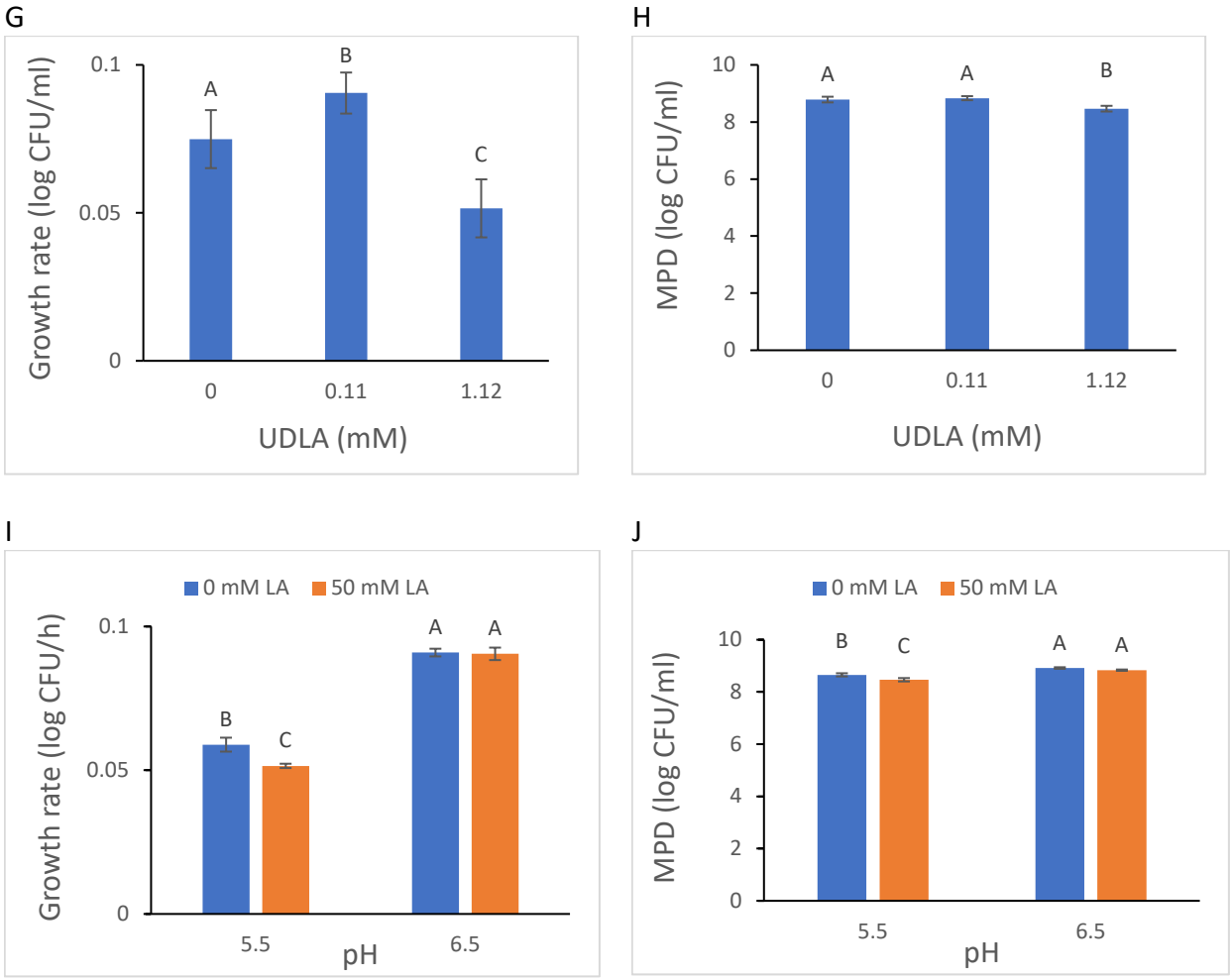


Figure 4. Effect of pH (A, B), lactic acid (C, D), glucose (E, F), UDLA (G, H) and the interaction between pH and lactic acid (I, J) on the GR and MPD of *S. liquefaciens*. Error bars represent the standard error of the mean. Means with the same letter in each panel were not significantly different.

Appendix B

Supplemental Data of Chapter 5

Table A.1 Growth kinetics for *B. thermosphacta* at pH 6.5

Treatment	Media no.	Species[1]	Trial	Lag time (h)	Average lag time	GR (log CFU/h)	Average GR	MPD (log CFU/ml)	Average MPD	Difference in[2]	GR	MPD
0 mM lactic acid [0mM UDLA]	1	Bt	1	3.20	1.60	0.096	0.090	7.40	7.59	Bt-(Bt/Cm/Sl)	-0.015	0.72
			2	0.00		0.084		7.78				
		Bt/Cm/Sl	1	5.33	5.61	0.103	0.105	6.40	6.88			
			2	5.88		0.107		7.35				
		Bt/Cm	not tested									
		Bt/Sl										
25 mM lactic acid [0.06 mM UDLA]	2	Bt	1	0.00	2.13	0.064	0.066	7.60	7.56	Bt-(Bt/Cm/Sl)	-0.012	0.60
			2	4.25		0.068		7.51				
		Bt/Cm/Sl	1	5.40	5.27	0.076	0.078	6.90	6.96			
			2	5.13		0.079		7.02				
		Bt/Cm	not tested									
		Bt/Sl										

50 mM lactic acid [0.11 mM UDLA]	3	Bt	1	7.48	7.91	0.059	0.059	7.30	7.43	Bt-(Bt/Cm/Sl)	0.008	1.04
			2	8.33		0.059		7.56				
		Bt/Cm/Sl	1	6.69	3.35	0.058	0.051	6.37	6.39	Bt-(Bt/Cm)	-0.009	1.14
			2	0.00		0.044		6.41				
		Bt/Cm	1	17.72	19.71	0.065	0.068	6.31	6.30	Bt-(Bt/Sl)	-0.005	0.58
			2	21.69		0.070		6.28				
		Bt/Sl	1	12.31	14.00	0.062	0.064	7.00	6.85	(Bt-(Bt/Cm))+(Bt-(Bt/Sl))	-0.014	1.72
			2	15.68		0.066		6.69				
75 mM lactic acid [0.17 mM UDLA]	4	Bt	1	13.98	9.59	0.044	0.044	7.00	7.17	Bt-(Bt/Cm/Sl)	-0.009	2.03
			2	5.20		0.044		7.34				
		Bt/Cm/Sl	1	17.09	8.55	0.052	0.053	5.00	5.14	Bt-(Bt/Cm)	0.013	1.07
			2	0.00		0.053		5.28				
		Bt/Cm	1	8.77	12.90	0.035	0.031	6.07	6.11	Bt-(Bt/Sl)	0.016	0.87
			2	17.02		0.027		6.14				
		Bt/Sl	1	0.00	3.87	0.031	0.029	6.31	6.31	(Bt-(Bt/Cm))+(Bt-(Bt/Sl))	0.029	1.93
			2	7.73		0.026		6.30				
100 mM lactic acid [0.22 mM UDLA]	5	Bt	1	64.04	56.15	0.0170	0.019	nd	7.19	Bt-(Bt/Cm/Sl)	0.005	2.37
			2	48.25		0.0200		7.19				
		Bt/Cm/Sl	1	18.49	48.58	0.0120	0.014	nd	4.82	Bt-(Bt/Cm)	0.000	1.99
			2	78.67		0.0160		4.82				
		Bt/Cm	1	14.98	16.78	0.022	0.019	5.12	5.20	Bt-(Bt/Sl)	0.002	1.71
			2	18.57		0.015		5.28				
		Bt/Sl	1	15.00	12.63	0.020	0.017	5.50	5.48	(Bt-(Bt/Cm))+(Bt-(Bt/Sl))	0.002	3.70
			2	10.26		0.013		5.46				

[1] Bt, *Brochothrix thermosphacta*; Cm, *Carnobacterium malartomaticum*, Sl, *Serratia liquefaciens*

[2] equation used to calculate difference in MPD between 1-. 2-, and 3-species systems

Table A.2 Growth kinetics for *B. thermosphacta* at pH 5.5

Treatment	Media no.	Species[1]	Trial	Lag time (h)	Average lag time	GR (log CFU/h)	Average GR	MPD (log CFU/ml)	Average MPD	Difference in[2]	GR	MPD
0 mM lactic acid [0 mM UDLA]	6	Bt	1	12.83	17.97	0.067	0.062	7.22	7.16	Bt-(Bt/Cm/Sl)	0.001	0.18
			2	23.11		0.057		7.11				
		Bt/Cm/Sl	1	10.89	17.92	0.064	0.061	7.06	6.98			
			2	24.94		0.059		6.90				
		Bt/Cm	not tested									
		Bt/Sl										
25 mM lactic acid [0.56 mM UDLA]	7	Bt	1	25.79	32.19	0.027	0.033	6.62	6.93	Bt-(Bt/Cm/Sl)	0.007	0.84
			2	38.59		0.039		7.24				
		Bt/Cm/Sl	1	16.43	24.44	0.027	0.027	5.99	6.08			
			2	32.45		0.026		6.18				
		Bt/Cm	not tested									
		Bt/Sl										
50 mM lactic acid [1.12 mM UDLA]	8	Bt	1	270.60	183.87	0.020	0.018	6.64	6.79	Bt-(Bt/Cm/Sl)	0.011	2.52
			2	97.13		0.016		6.93				
		Bt/Cm/Sl	1	118.90	273.20	0.006	0.007	4.45	4.26	Bt-(Bt/Cm)	0.016	3.59
			2	427.50		0.009		4.08				
		Bt/Cm	1	0.00	0.00	0.001	0.001	3.31	3.20	Bt-(Bt/Sl)	0.007	2.18

Treatment	Media no.	Species[1]	Trial	Lag time (h)	Average lag time	GR (log CFU/h)	Average GR	MPD (log CFU/ml)	Average MPD	Difference in[2]	GR	MPD
			2	0.00		0.002		3.08				
		Bt/Sl	1	323.70	193.46	0.018	0.011	4.79	4.61	(Bt-(Bt/Cm))+(Bt-(Bt/Sl))	0.023	5.77
			2	63.21		0.004		4.42				
75 mM lactic acid [1.68 mM UDLA]	9	Bt	1	20.780	1172.39	ng[3]						
			2	2324.000								
		Bt/Cm/Sl	1	767.600	794.50							
			2	821.400								
		Bt/Cm	1	369.800	474.50							
			2	579.200								
		Bt/Sl	1	165.200	482.15							
			2	799.100								
100 mM lactic acid [2.24 mM UDLA]	10	Bt	1	59.25	303.43	ng						
			2	547.60								
		Bt/Cm/Sl	1	1081.00	1134.50							
			2	1188.00								
		Bt/Cm	not tested									
		Bt/Sl										

[1] Bt, *Brochothrix thermosphacta*; Cm, *Carnobacterium maltaromaticum*, Sl, *Serratia liquefaciens*

[2] equation used to calculate difference in MPD between 1-. 2-, and 3-species systems

[3] ng, growth was not observed

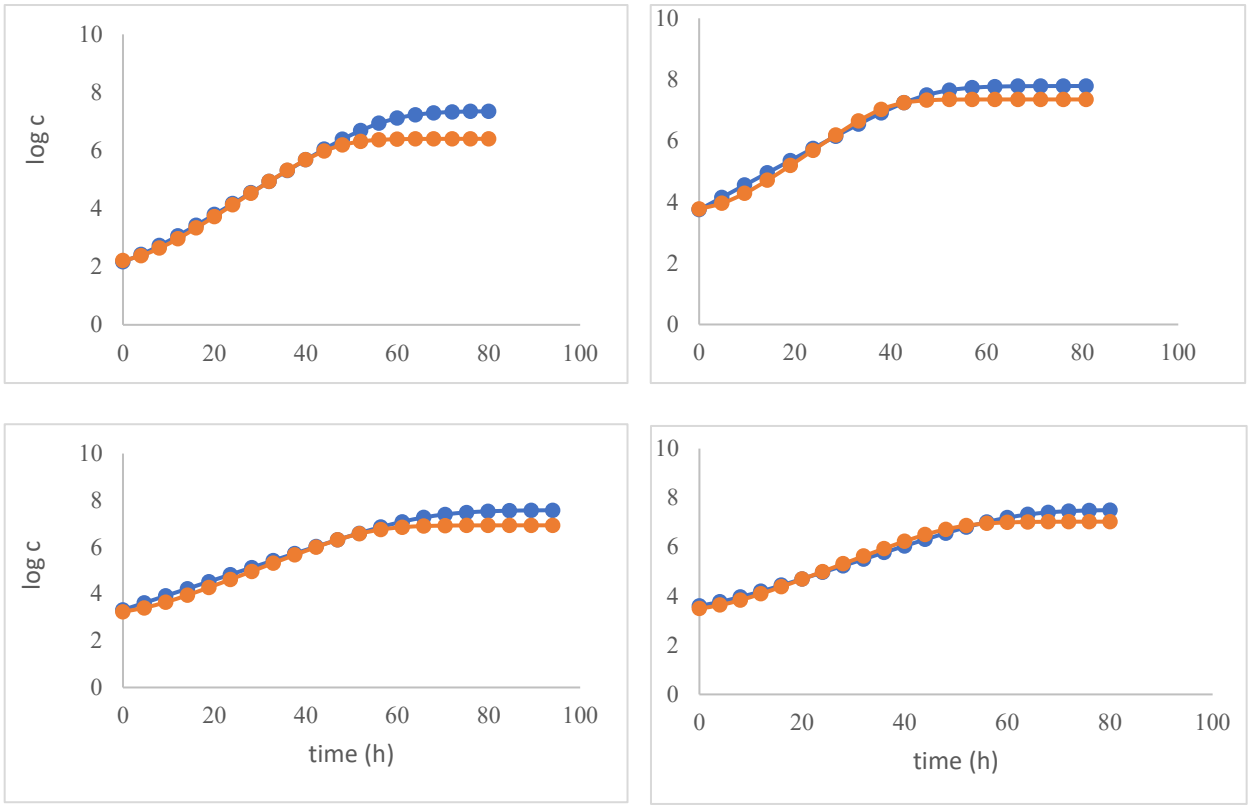


Figure A.1 *B. thermosphacta* grown in 0.00 mM (top panels) and 0.06 mM UDLA (bottom panels) at pH 6.5 as individual and 3-species mixed culture, for trials 1 (left panels) and 2 (right panels).

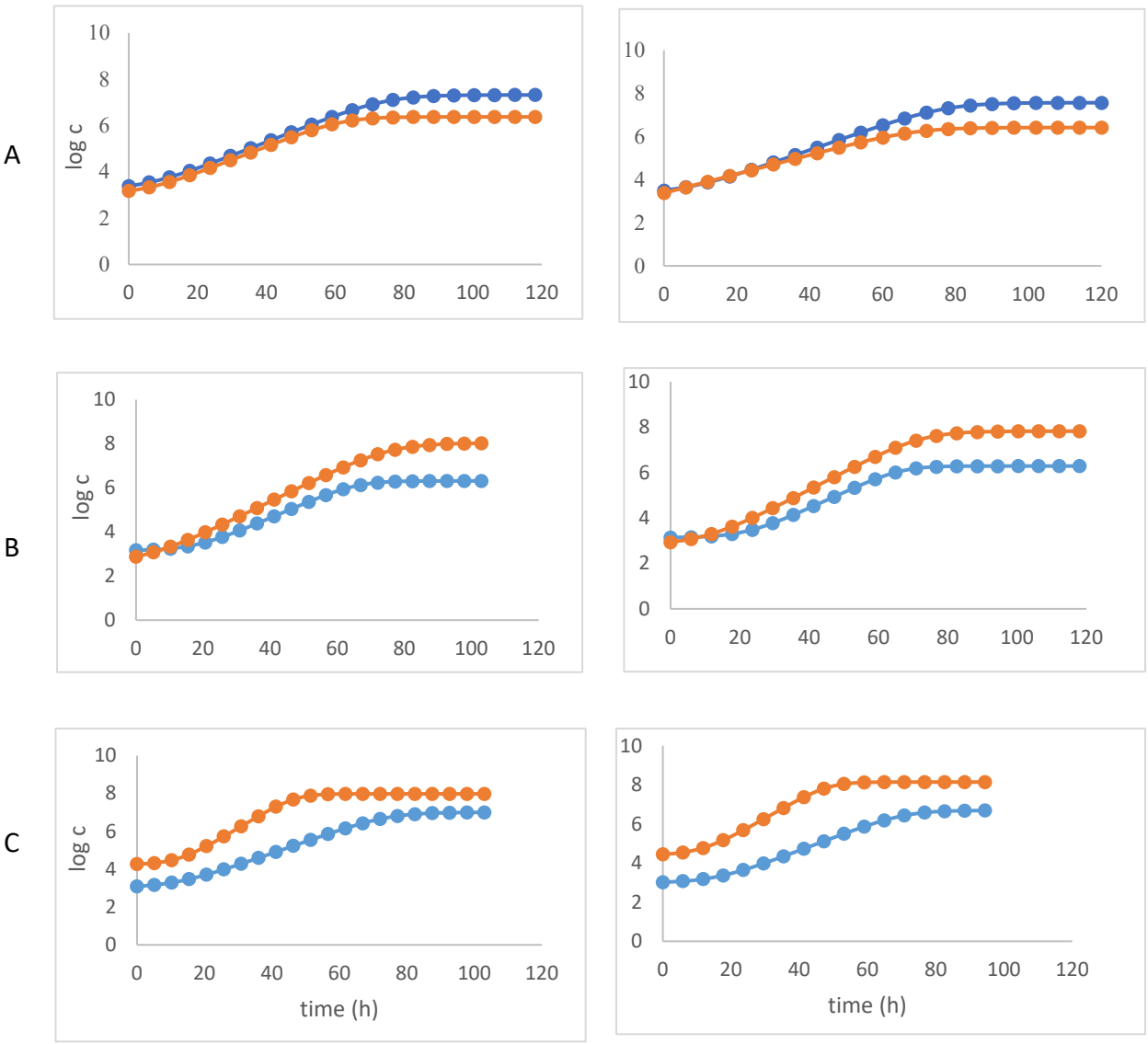


Figure A.2 (A) *B. thermosphacta* grown in 0.11 mM UDLA at pH 6.5 as single (blue line) and in 3-species culture (orange line), (B) *B. thermosphacta* (blue line) co-cultured with C. *maltaromaticum* (orange line), and (C) *B. thermosphacta* (blue line) co-cultured with *S. liquefaciens* (orange line), for trials 1 (left panels) and 2 (right panels).

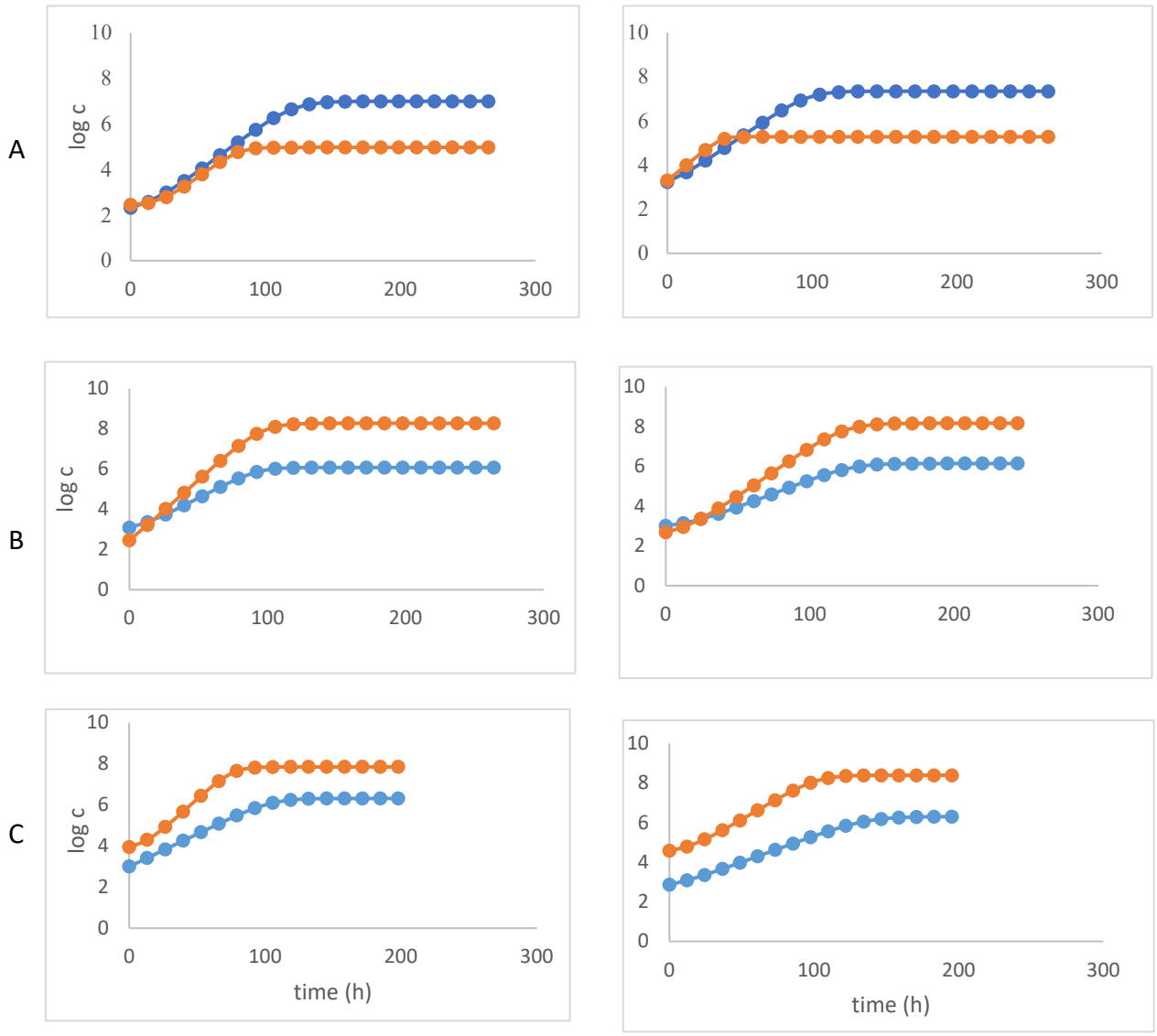


Figure A.3 (A) *B. thermosphacta* grown in 0.17 mM UDLA at pH 6.5 as single (blue line) and in 3-species culture (orange line), (B) *B. thermosphacta* (blue line) co-cultured with C. *maltaromaticum* (orange line), and (C) *B. thermosphacta* (blue line) co-cultured with *S. liquefaciens* (orange line), for trials 1 (left panels) and 2 (right panels).

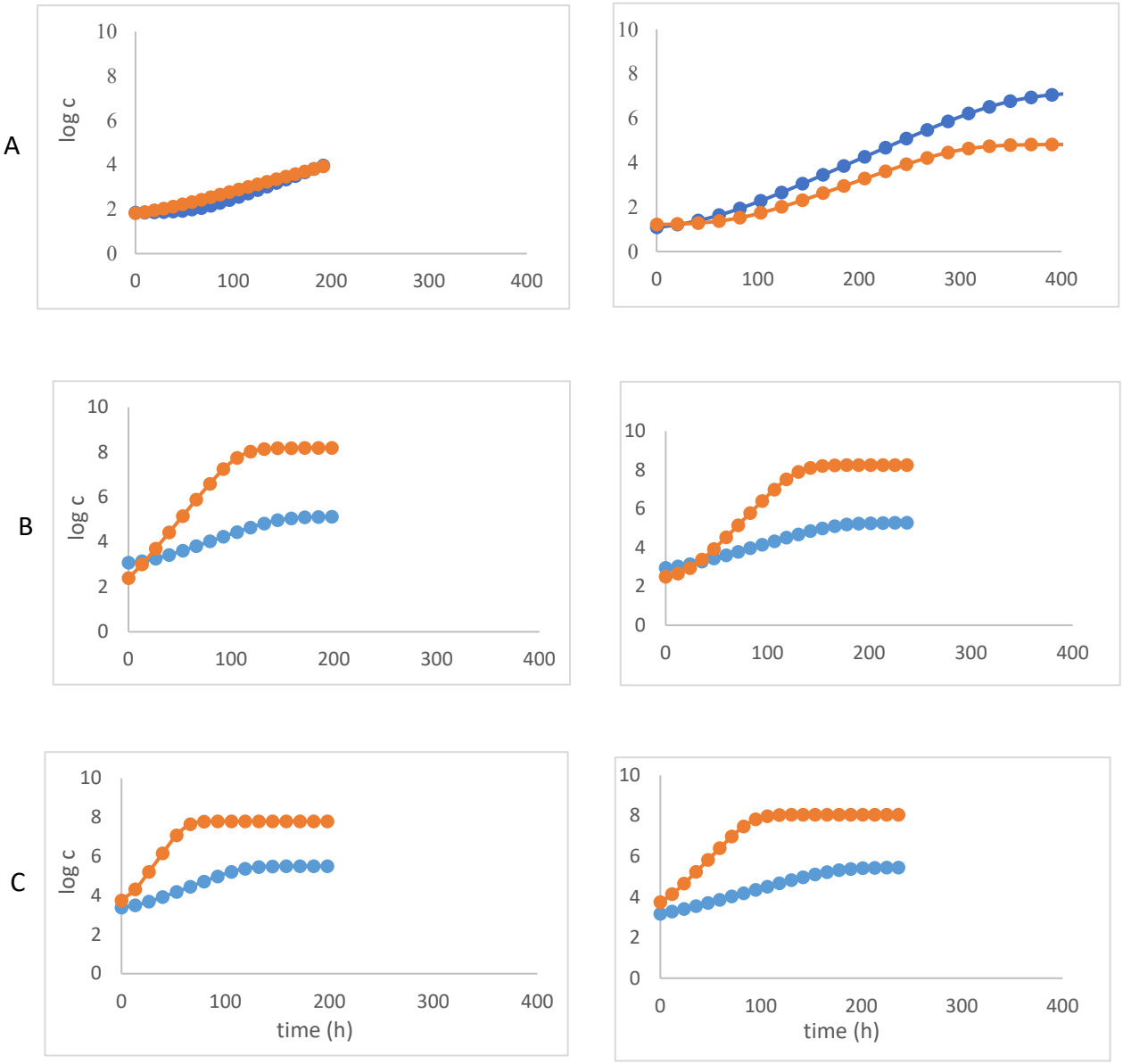


Figure A.4 (A) *B. thermosphacta* grown in 0.22 mM UDLA at pH 6.5 as single (blue line) and in 3-species culture (orange line), (B) *B. thermosphacta* (blue line) co-cultured with C*. maltaromaticum* (orange line), and (C) *B. thermosphacta* (blue line) co-cultured with *S. liquefaciens* (orange line), for trials 1 (left panels) and 2 (right panels).

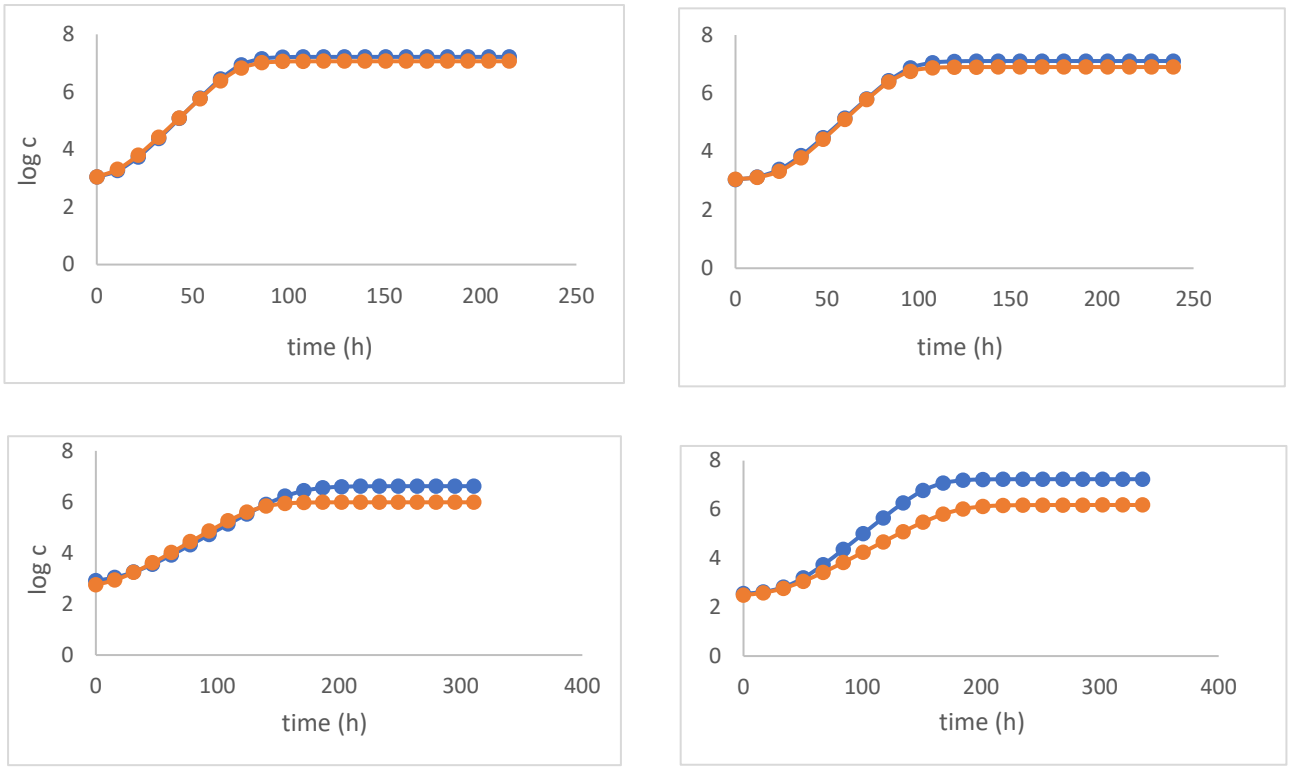


Figure A.5 *B. thermosphacta* grown in 0.00 mM (top panels) and 0.56 mM UDLA (bottom panels) at pH 5.5 as individual and 3-species mixed culture, for trials 1 (left panels) and 2 (right panels)

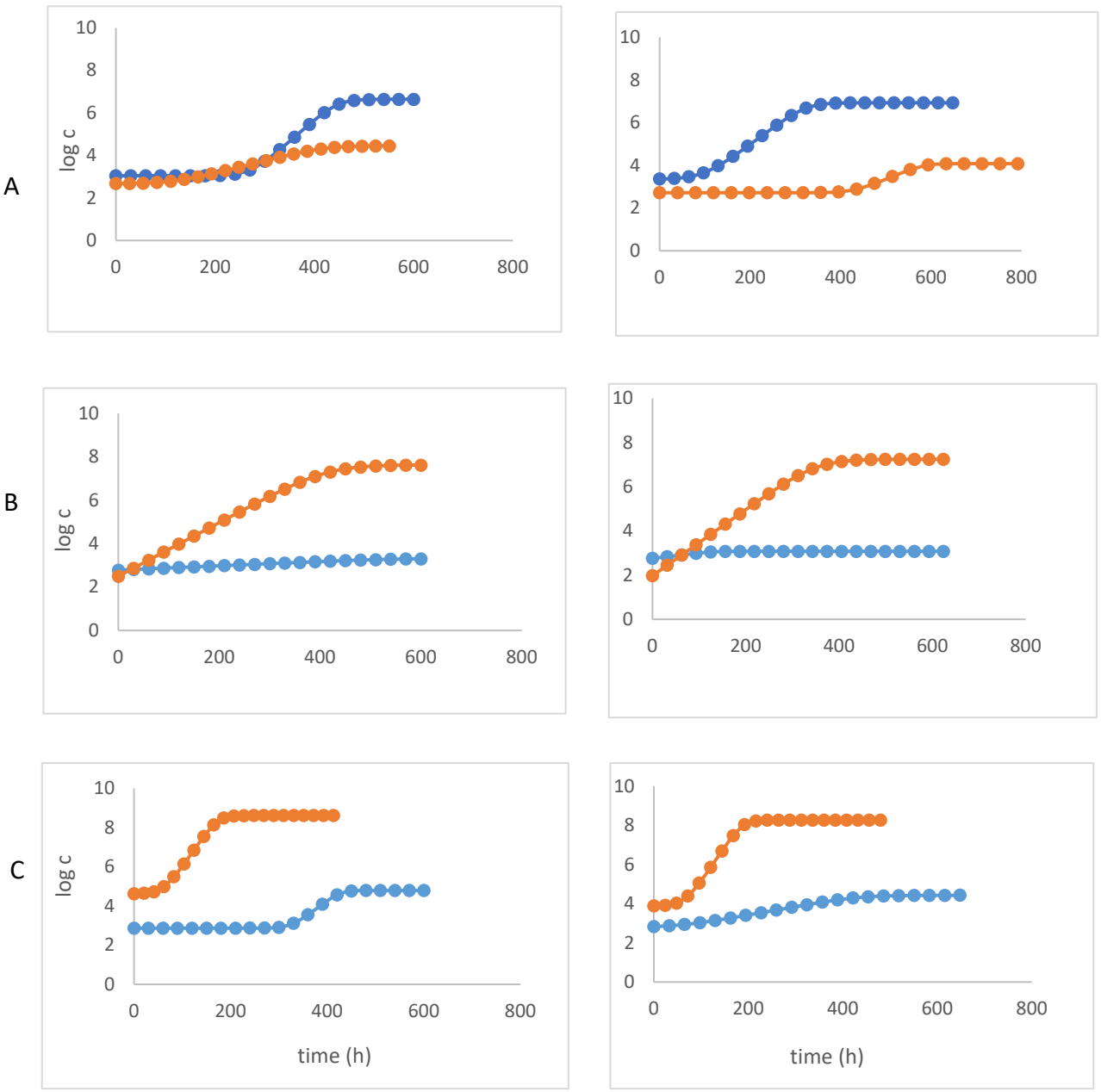


Figure A.6 (A) *B. thermosphacta* grown in 1.12 mM UDLA at pH 5.5 as single (blue line) and in 3-species culture (orange line), (B) *B. thermosphacta* (blue line) co-cultured with C. *maltaromaticum* (orange line), and (C) *B. thermosphacta* (blue line) co-cultured with *S. liquefaciens* (orange line), for trials 1 (left panels) and 2 (right panels).

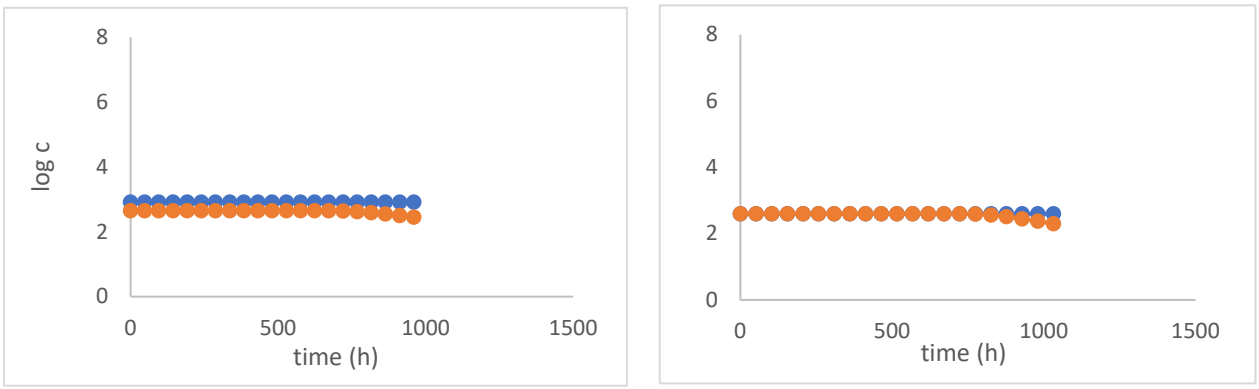


Figure A.7 *B. thermosphacta* grown in 1.68 mM UDLA at pH 5.5 as individual (blue line) and in 3-species culture (orange line), for trials 1 (left panel) and 2 (right panel).

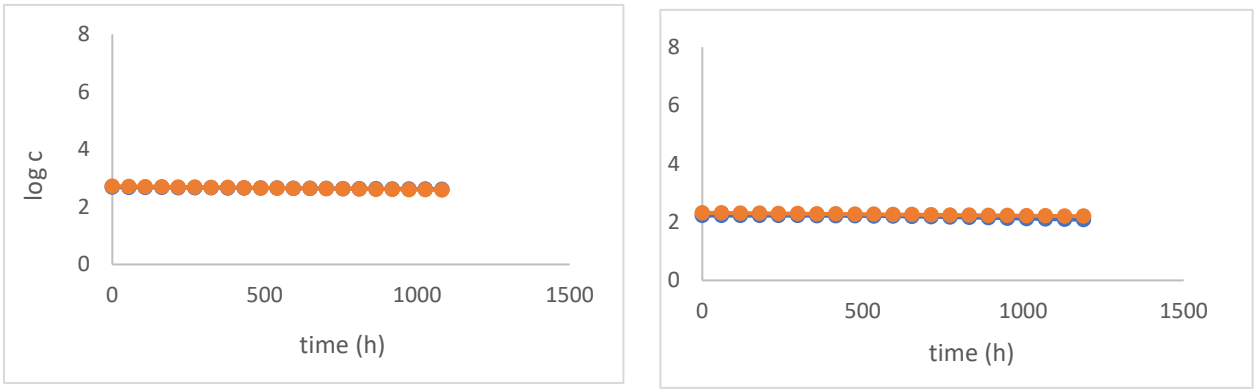


Figure A.8 *B. thermosphacta* grown in 2.24 mM UDLA at pH 5.5 as individual (blue line) and in 3-species culture (orange line), for trials 1 (left panel) and 2 (right panel).

Supplemental Data

Table B.1 Growth kinetics for *C. maltaromaticum* at pH 6.5

Treatment	Media no.	Species[1]	Trial	Lag time (h)	Average lag time	GR (log CFU/h)	Average GR	MPD (log CFU/ml)	Average MPD	Difference in[2]	GR	MPD
0 mM lactic acid [0 mM UDLA]	1	Cm	1	3.01	5.65	0.089	0.089	8.40	8.34	Cm-(Bt/Cm/Sl)	0.004	0.94
			2	8.28		0.088		8.28				
		Bt/Cm/Sl	1	3.61	5.51	0.085	0.084	7.40	7.40			
			2	7.40		0.083		7.40				
		Bt/Cm	not tested									
		Cm/Sl										
25 mM lactic acid [0.06 mM UDLA]	2	Cm	1	3.81	1.91	0.086	0.083	8.15	8.29	Cm-(Bt/Cm/Sl)	-0.015	0.62
			2	0.00		0.079		8.42				
		Bt/Cm/Sl	1	5.27	6.46	0.089	0.098	7.89	7.67			
			2	7.65		0.106		7.45				
		Bt/Cm	not tested									
		Cm/Sl										
50 mM lactic acid [0.11 mM UDLA]	3	Cm	1	7.25	7.68	0.082	0.081	8.09	8.04	Cm-(Bt/Cm/Sl)	0.016	0.56
			2	8.11		0.080		7.99				
		Bt/Cm/Sl	1	2.70	2.25	0.070	0.065	7.52	7.49	Cm-(Bt/Cm)	0.005	0.11
			2	1.79		0.060		7.45				

		Bt/Cm	1	6.40	8.70	0.074	0.077	8.03	7.93	Cm-(Bt/Sl)	0.012	0.20
			2	10.99		0.079		7.82				
		Cm/Sl	1	4.39	5.47	0.069	0.069	7.98	7.84	(Cm-(Bt/Cm))+(Cm-(Cm/Sl))	0.017	0.31
			2	6.55		0.069		7.70				
75 mM lactic acid [0.17 mM UDLA]	4	Cm	1	8.05	4.03	0.075	0.072	8.00	8.22	Cm-(Bt/Cm/Sl)	-0.014	0.25
			2	0.00		0.068		8.43				
		Bt/Cm/Sl	1	11.53	6.99	0.091	0.086	7.75	7.97	Cm-(Bt/Cm)	0.016	-0.01
			2	2.45		0.080		8.19				
		Bt/Cm	1	1.27	7.69	0.061	0.056	8.28	8.22	Cm-(Bt/Sl)	0.014	0.32
			2	14.11		0.050		8.17				
		Cm/Sl	1	4.11	6.38	0.066	0.058	7.96	7.89	(Cm-(Bt/Cm))+(Cm-(Cm/Sl))	0.030	0.31
			2	8.66		0.050		7.82				
100 mM lactic acid [0.22 mM UDLA]	5	Cm	1	8.03	9.39	0.0550	0.054	8.08	8.02	Cm-(Bt/Cm/Sl)	-0.006	0.21
			2	10.75		0.0530		7.95				
		Bt/Cm/Sl	1	6.71	3.36	0.0590	0.060	7.91	7.81	Cm-(Bt/Cm)	-0.001	-0.20
			2	0.00		0.0600		7.71				
		Bt/Cm	1	3.29	12.77	0.055	0.055	8.19	8.22	Cm-(Bt/Sl)	0.010	0.17
			2	22.26		0.054		8.25				
		Cm/Sl	1	0.00	4.53	0.047	0.044	7.96	7.85	(Cm-(Bt/Cm))+(Cm-(Cm/Sl))	0.010	-0.03
			2	9.06		0.041		7.73				

[1] Bt, *Brochothrix thermosphacta*; Cm, *Carnobacterium malartomaticum*, Sl, *Serratia liquefaciens*

[2] equation used to calculate difference in MPD between 1-. 2-, and 3-species systems

Table B.2 Growth kinetics for *C. maltaromaticum* at pH 5.5

Treatment	Media no.	Species[1]	Trial	Lag time (h)	Average lag time	GR (log CFU/h)	Average GR	MPD (log CFU/ml)	Average MPD	Difference in[2]	GR	MPD
0 mM lactic acid [0 mM UDLA]	6	Cm	1	5.29	2.65	0.060	0.056	8.41	8.13	Cm-(Bt/Cm/Sl)	-0.007	0.11
			2	0.00		0.052		7.85				
		Bt/Cm/Sl	1	3.37	14.52	0.058	0.063	8.15	8.03			
			2	25.67		0.068		7.90				
		Bt/Cm	not tested									
		Cm/Sl										
25 mM lactic acid [0.56 mM UDLA]	7	Cm	1	5.39	2.70	0.044	0.041	7.95	8.00	Cm-(Bt/Cm/Sl)	-0.003	0.01
			2	0.00		0.039		8.06				
		Bt/Cm/Sl	1	3.42	6.06	0.043	0.044	7.88	8.00			
			2	8.70		0.045		8.12				
		Bt/Cm	not tested									
		Cm/Sl										
50 mM lactic acid [1.12 mM UDLA]	8	Cm	1	35.41	17.71	0.018	0.016	7.90	7.78	Cm-(Bt/Cm/Sl)	-0.004	-0.20
			2	0.00		0.014		7.65				
		Bt/Cm/Sl	1	0.00	0.00	0.018	0.020	8.34	7.98	Cm-(Bt/Cm)	0.002	0.34
			2	0.00		0.022		7.62				
		Bt/Cm	1	0.00	0.00	0.012	0.014	7.63	7.43	Cm-(Bt/Sl)	-0.002	0.20
			2	0.00		0.015		7.24				
		Cm/Sl	1	0.00	28.15	0.016	0.018	7.85	7.58	(Cm-(Bt/Cm))+(Cm-(Cm/Sl))	0.000	0.54

			2	56.30		0.020		7.31				
75 mM lactic acid [1.68 mM UDLA]	9	Cm	1	38.05	19.03	0.000	0.000	2.32	2.23	Cm-(Bt/Cm/Sl)	-0.014	-4.81
			2	0.00		0.000		2.13				
		Bt/Cm/Sl	1	478.40	486.95	0.012	0.015	7.20	7.04	Cm-(Bt/Cm)	0.008	0.29
			2	495.50		0.017		6.88				
		Bt/Cm	1	71.73	270.17	-0.016	-0.008	1.85	1.94	Cm-(Bt/Sl)	0.000	-0.02
			2	468.60		0.000		2.03				
		Cm/Sl	1	170.90	361.75	0.000	0.000	2.19	2.25	(Cm-(Bt/Cm))+(Cm-(Cm/Sl))	0.008	0.27
			2	552.60		0.001		2.31				
100 mM lactic acid [2.24 mM UDLA]	10	Cm	1	0.02	20.52	ng[3]						
			2	41.02								
		Bt/Cm/Sl	1	4938.00	2469.00							
			2	0.00								
		Bt/Cm	not tested									
		Cm/Sl										

[1] Bt, *Brochothrix thermosphacta*; Cm, *Carnobacterium malartomaticum*, Sl, *Serratia liquefaciens*

[2] equation used to calculate difference in MPD between 1-. 2-, and 3-species systems

[3] ng, growth not observed

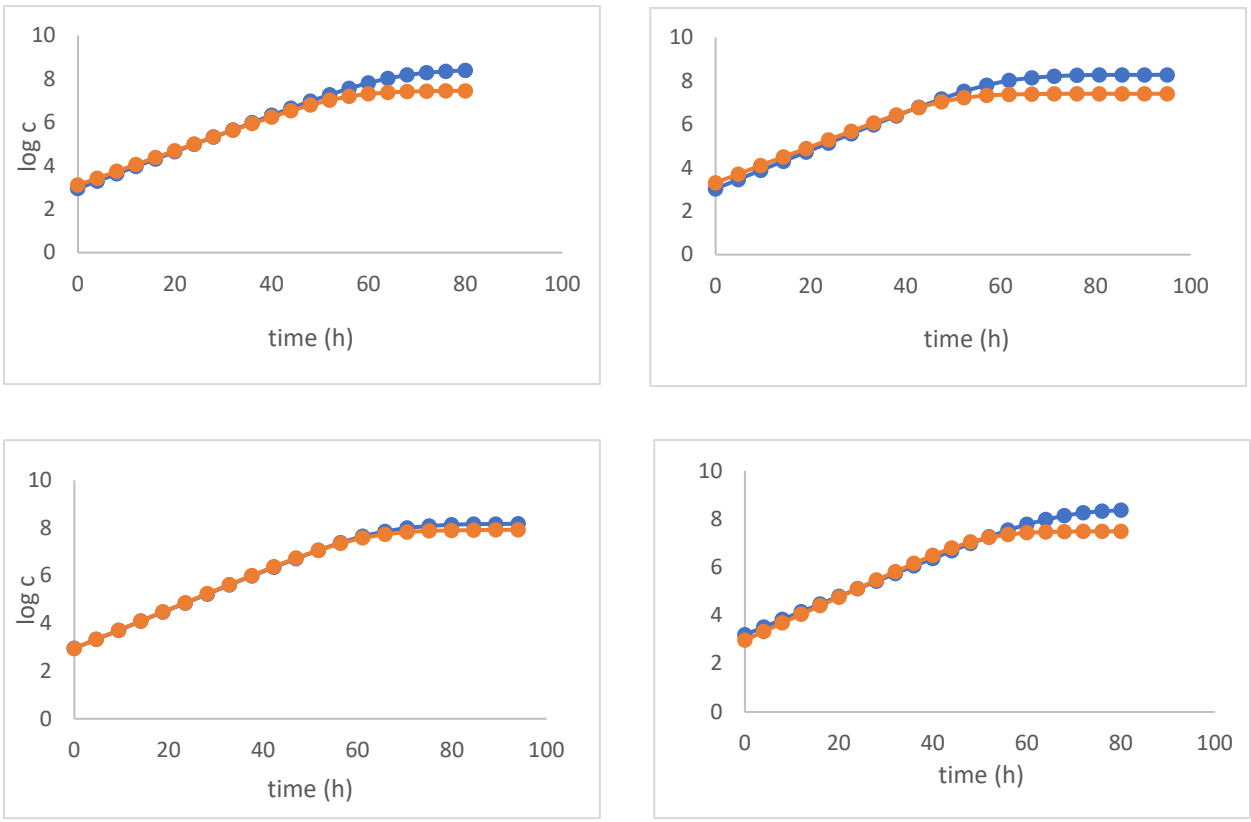


Figure B.1 *C. maltaromaticum* grown in 0.00 mM (top panels) and 0.06 mM UDLA (bottom panels) at pH 6.5 as individual and 3-species mixed culture, for trials 1 (left panels) and 2 (right panels).

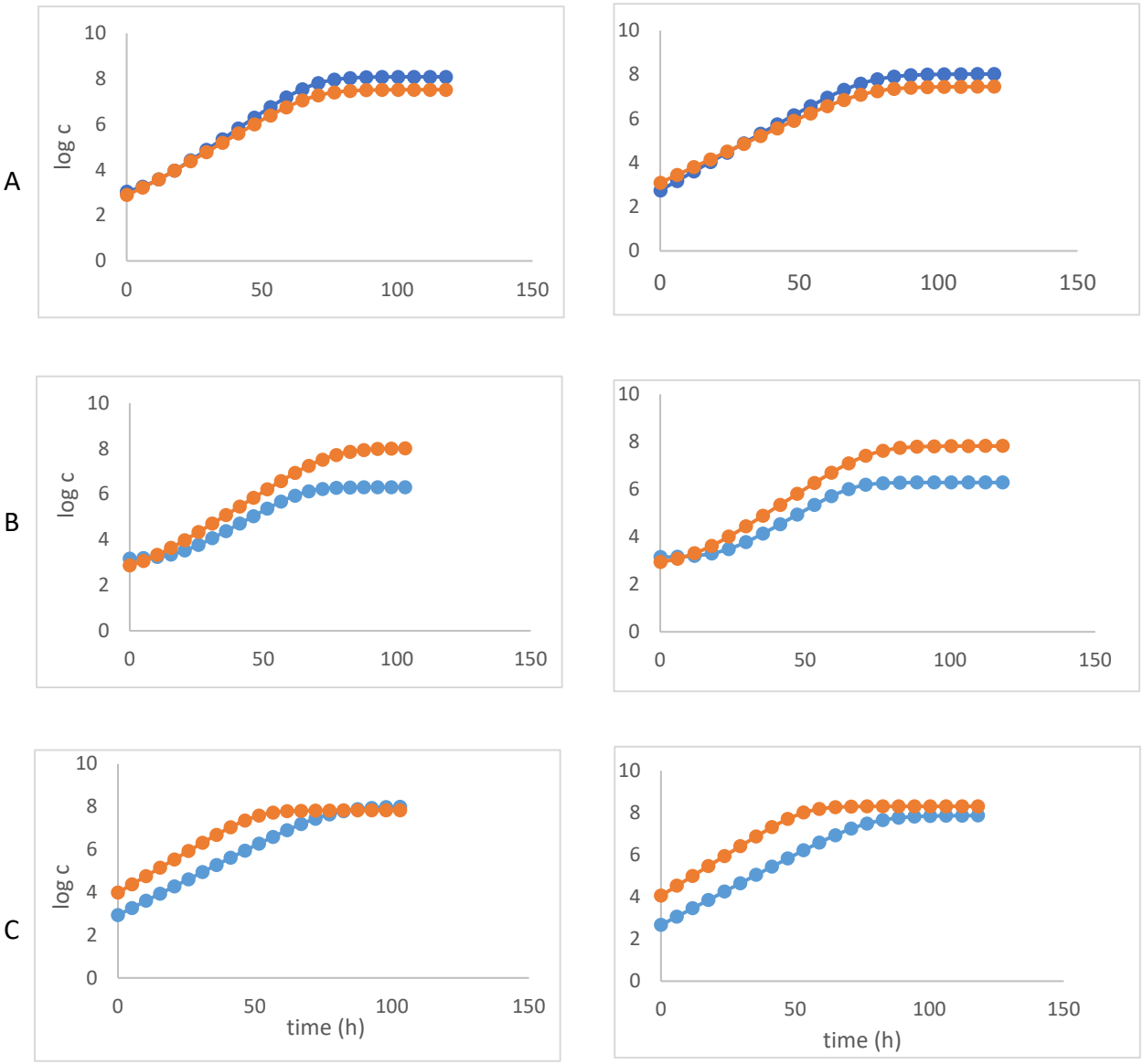


Figure B.2 (A) *C. maltaromaticum* grown in 0.11 mM UDLA at pH 6.5 as single (blue line) and in 3-species culture (orange line), (B) C*. maltaromaticum* (orange line) co-cultured with *B. thermosphacta* (blue line), and (C) C*. maltaromaticum* (blue line) co-cultured with *S. liquefaciens* (orange line), for trials 1 (left panels) and 2 (right panels).

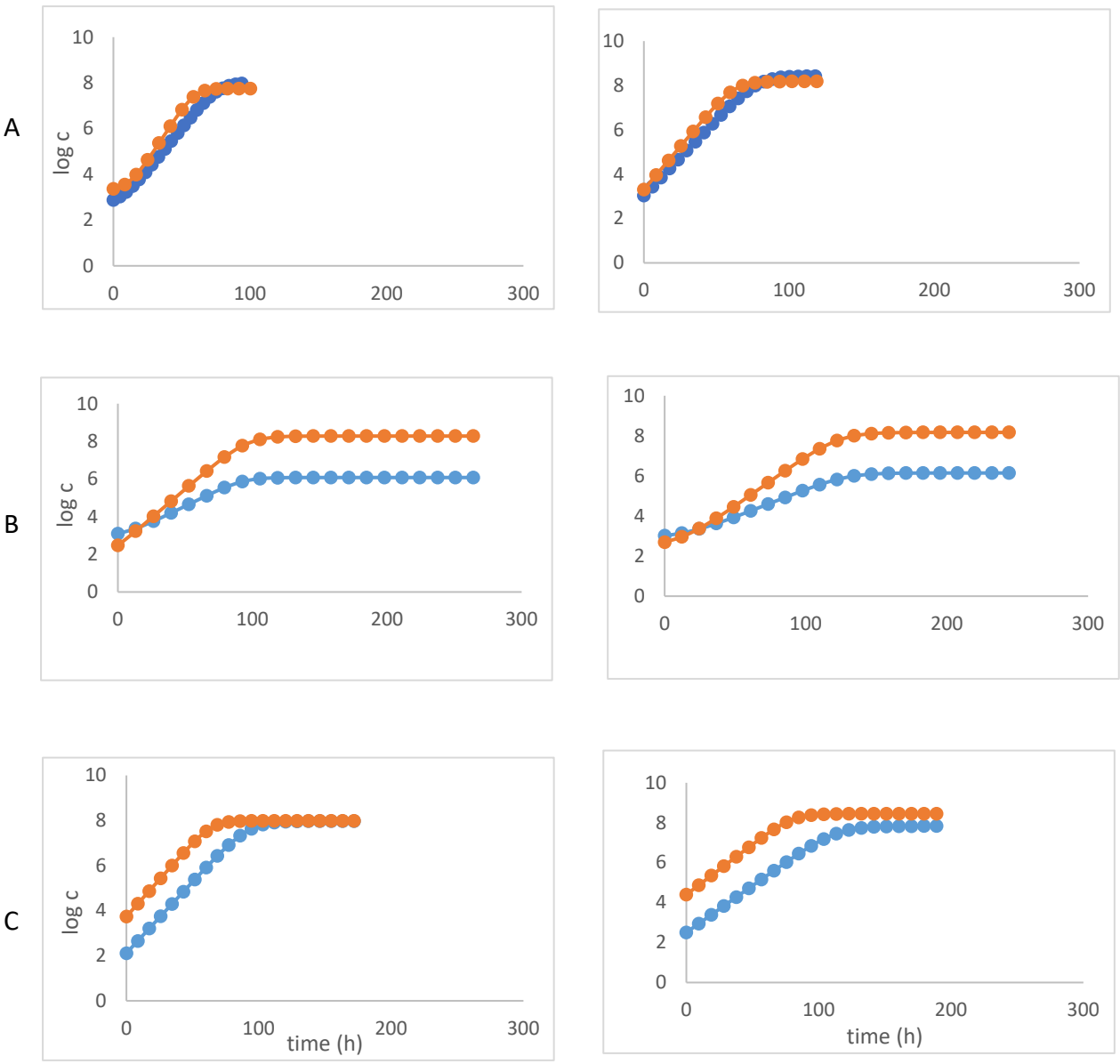


Figure B.3 (A) *C. maltaromaticum* grown in 0.17 mM UDLA at pH 6.5 as single (blue line) and in 3-species culture (orange line), (B) C*. maltaromaticum* (orange line) co-cultured with *B. thermosphacta* (blue line), and (C) C*. maltaromaticum* (blue line) co-cultured with *S. liquefaciens* (orange line), for trials 1 (left panels) and 2 (right panels).

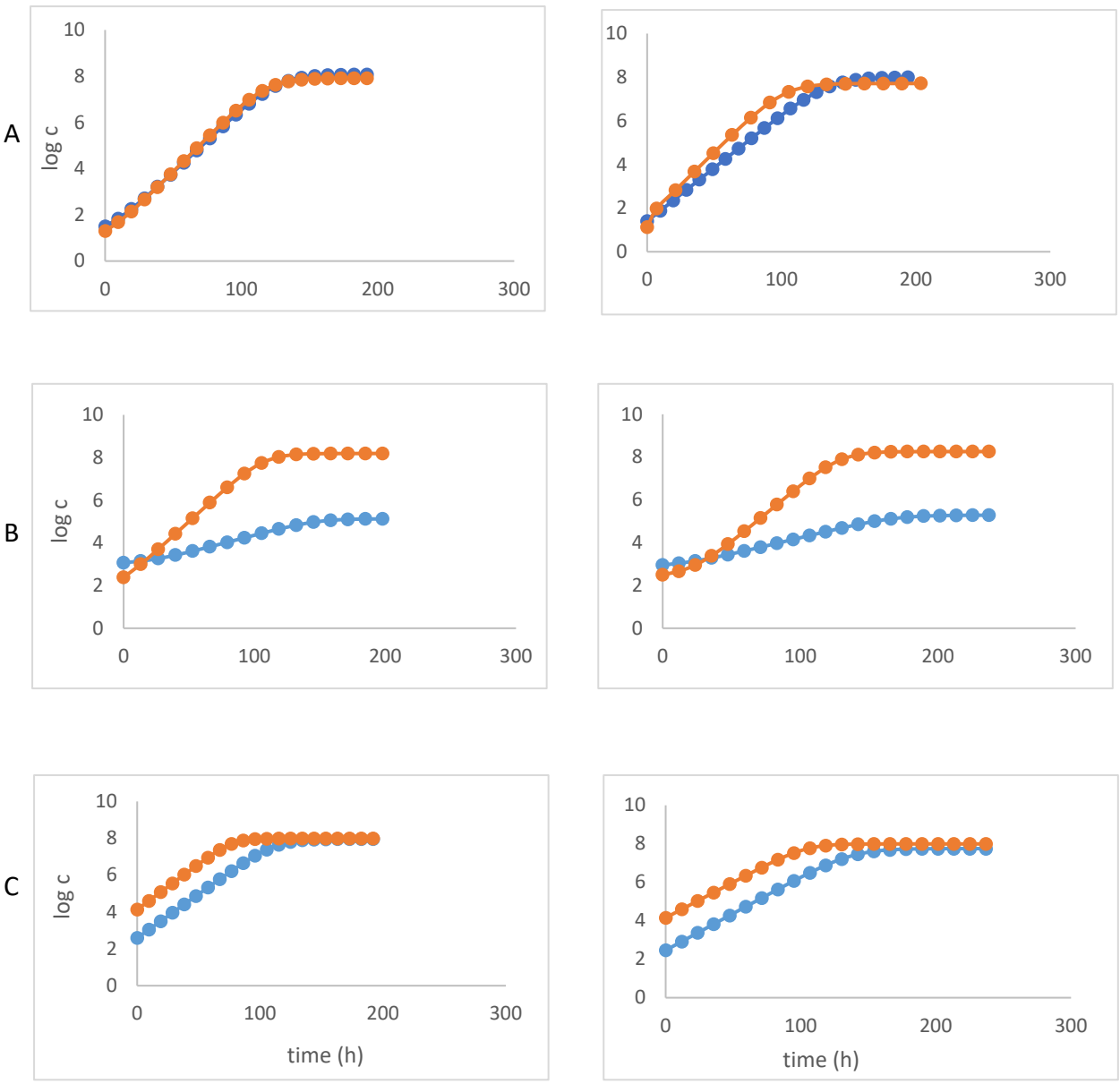


Figure B.4 (A) *C. maltaromaticum* grown in 0.22 mM UDLA at pH 6.5 as single (blue line) and in 3-species culture (orange line), (B) C*. maltaromaticum* (orange line) co-cultured with *B. thermosphacta* (blue line), and (C) C*. maltaromaticum* (blue line) co-cultured with *S. liquefaciens* (orange line), for trials 1 (left panels) and 2 (right panels).

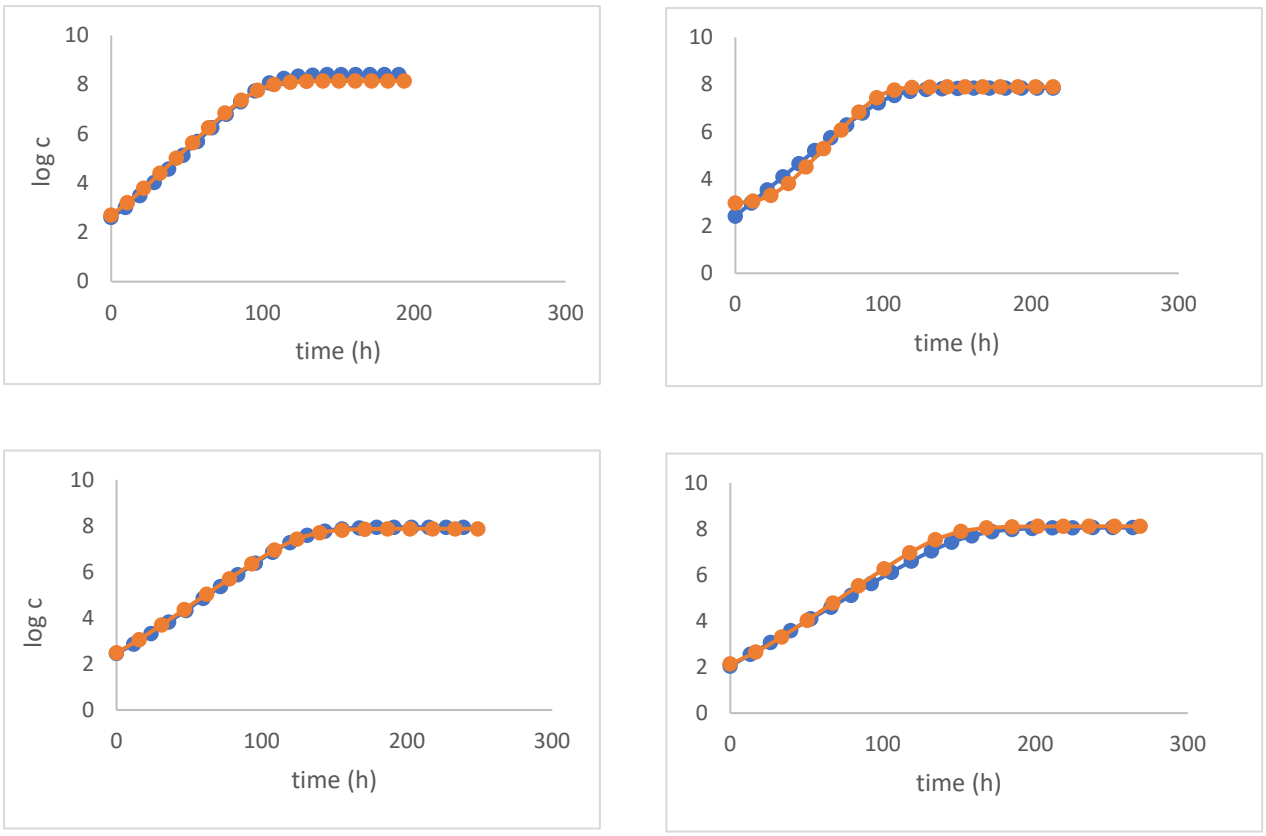


Figure B.5 *C. maltaromaticum* grown in 0.00 mM (top panels) and 0.56 mM (bottom panels) at pH 5.5 as individual and 3-species mixed culture, for trials 1 (left panels) and 2 (right panels).

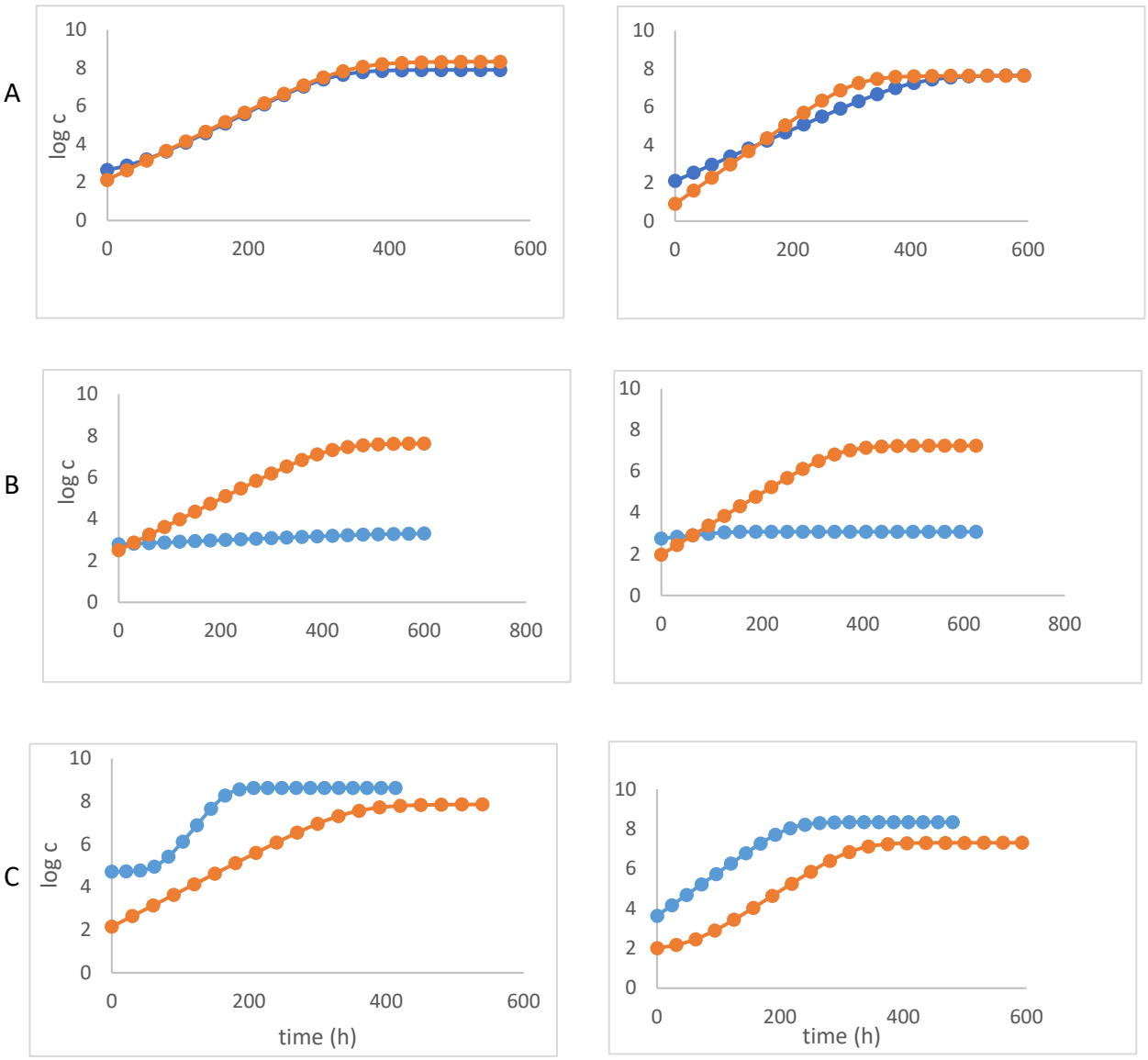


Figure B.6 (A) *C. maltaromaticum* grown in 1.12 mM UDLA at pH 5.5 as single (blue line) and in 3-species culture (orange line), (B) C*. maltaromaticum* (orange line) co-cultured with *B. thermosphacta* (blue line), and (C) C*. maltaromaticum* (orange line) co-cultured with *S. liquefaciens* (blue line), for trials 1 (left panels) and 2 (right panels).

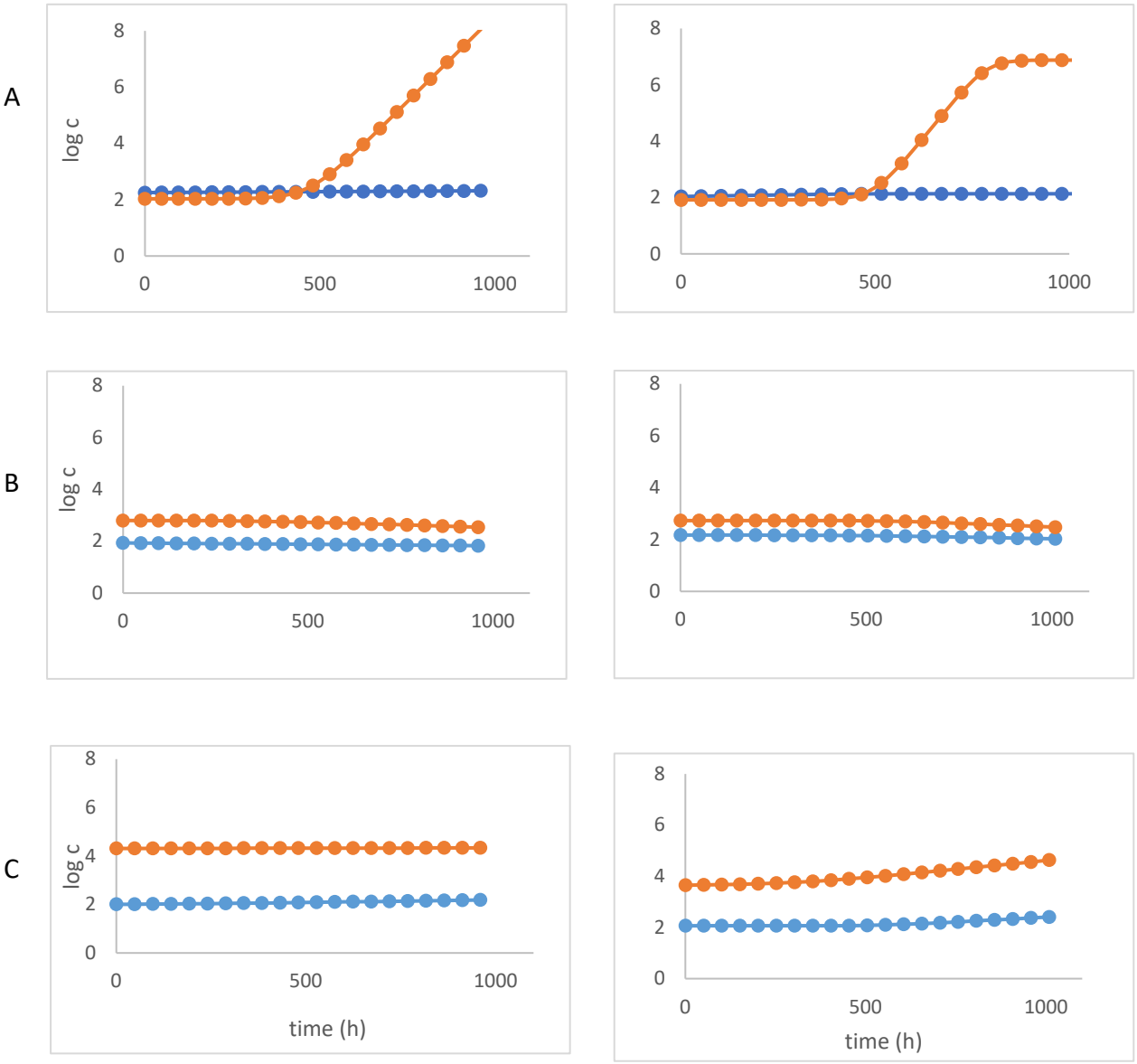


Figure B.7 (A) *C. maltaromaticum* grown in 1.68 mM UDLA at pH 5.5 as single (blue line) and in 3-species culture (orange line), (B) C*. maltaromaticum* (blue line) co-cultured with *B. thermosphacta* (orange line), and (C) C*. maltaromaticum* (blue line) co-cultured with *S. liquefaciens* (orange line), for trials 1 (left panels) and 2 (right panels).

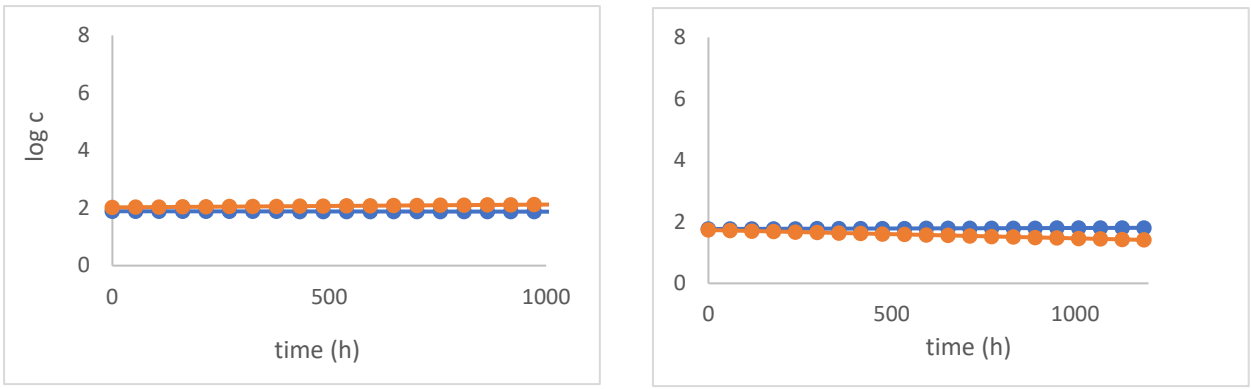


Figure B.8 *C. maltaromaticum* grown in 2.24 mM UDLA at pH 5.5 as single (blue line) and in 3-species culture (orange line), for trials 1 (left panels) and 2 (right panels).

Supplemental Data

Table C.1 Growth kinetics for *S. liquefaciens* at pH 6.5

Treatment	Media no.	Species[1]	Trial	Lag time (h)	Average lag time	GR (log CFU/h)	Average GR	MPD (log CFU/ml)	Average MPD	Difference in[2]	GR	MPD
0 mM lactic acid [0 mM UDLA]	1	Sl	1	7.20	6.27	0.110	0.120	8.46	8.31	Sl-(Bt/Cm/Sl)	-0.010	0.32
			2	5.33		0.130		8.16				
		Bt/Cm/Sl	1	10.50	9.57	0.120	0.130	8.21	8.00			
			2	8.63		0.140		7.78				
		Bt/Sl	not tested									
		Cm/Sl										
25 mM lactic acid [0.06 mM UDLA]	2	Sl	1	10.37	8.69	0.100	0.115	8.39	8.46	Sl-(Bt/Cm/Sl)	0.019	0.02
			2	7.01		0.130		8.52				
		Bt/Cm/Sl	1	5.91	4.40	0.093	0.097	8.37	8.44			
			2	2.88		0.100		8.50				
		Bt/Sl	not tested									
		Cm/Sl										

50 mM lactic acid [0.11 mM UDLA]	3	Sl	1	14.25	9.02	0.140	0.120	8.20	8.20	Sl-(Bt/Cm/Sl)	0.005	0.49
			2	3.78		0.100		8.19				
		Bt/Cm/Sl	1	9.34	7.99	0.120	0.115	7.80	7.71	Sl-(Bt/Sl)	0.021	0.14
			2	6.63		0.110		7.62				
		Bt/Sl	1	12.20	11.88	0.100	0.100	7.97	8.06	Sl-(Cm/Sl)	0.015	0.14
			2	11.55		0.099		8.14				
		Cm/Sl	1	14.25	13.36	0.110	0.105	7.82	8.06	(Sl-(Bt/Sl))+(Sl-(Cm/Sl))	0.036	0.28
			2	12.46		0.100		8.30				
75 mM lactic acid [0.17 mM UDLA]	4	Sl	1	4.62	2.31	0.079	0.079	8.02	7.87	Sl-(Bt/Cm/Sl)	-0.012	-0.07
			2	0.00		0.079		7.72				
		Bt/Cm/Sl	1	2.95	1.48	0.085	0.091	7.84	7.94	Sl-(Bt/Sl)	0.012	-0.25
			2	0.00		0.096		8.03				
		Bt/Sl	1	7.63	9.05	0.081	0.067	7.84	8.12	Sl-(Cm/Sl)	0.012	-0.34
			2	10.47		0.053		8.39				
		Cm/Sl	1	9.60	4.80	0.085	0.068	7.96	8.21	(Sl-(Bt/Sl))+(Sl-(Cm/Sl))	0.024	-0.58
			2	0.00		0.050		8.45				
100 mM lactic acid [0.22 mM UDLA]	5	Sl	1	30.82	27.18	0.043	0.048	8.10	8.35	Sl-(Bt/Cm/Sl)	0.004	0.23
			2	23.53		0.052		8.60				
		Bt/Cm/Sl	1	28.23	25.45	0.038	0.044	7.90	8.12	Sl-(Bt/Sl)	-0.008	0.43
			2	22.67		0.049		8.33				
		Bt/Sl	1	7.02	6.61	0.061	0.056	7.79	7.92	Sl-(Cm/Sl)	0.002	0.37
			2	6.19		0.050		8.05				
		Cm/Sl	1	3.28	9.11	0.049	0.046	7.98	7.98	(Sl-(Bt/Sl))+(Sl-(Cm/Sl))	-0.007	0.80
			2	14.94		0.043		7.98				

[1] Bt, *Brochothrix thermosphacta*; Cm, *Carnobacterium malartomaticum*, Sl, *Serratia liquefaciens*

[2] equation used to calculate difference in MPD between 1-. 2-, and 3-species systems

Table C.2 Growth kinetics for *S. liquefaciens* at pH 5.5

Treatment	Media no.	Species[1]	Trial	Lag time (h)	Average lag time	GR (log CFU/h)	Average GR	MPD (log CFU/ml)	Average MPD	Difference in[2]	GR	MPD
0 mM lactic acid [0 mM UDLA]	6	Sl	1	21.63	19.68	0.075	0.070	8.48	8.39	Sl-(Bt/Cm/Sl)	0.010	0.15
			2	17.72		0.066		8.31				
		Bt/Cm/Sl	1	14.54	18.69	0.069	0.060	8.33	8.25			
			2	22.83		0.052		8.17				
		Bt/Sl	not tested									
		Cm/Sl										
25 mM lactic acid [0.56 mM UDLA]	7	Sl	1	15.01	25.21	0.043	0.045	7.80	7.91	Sl-(Bt/Cm/Sl)	0.002	-0.16
			2	35.40		0.047		8.02				
		Bt/Cm/Sl	1	18.16	20.93	0.053	0.043	8.02	8.07			
			2	23.70		0.033		8.12				
		Bt/Sl	not tested									
		Cm/Sl										
50 mM lactic acid [1.12 mM UDLA]	8	Sl	1	34.30	48.43	0.029	0.022	7.85	8.04	Sl-(Bt/Cm/Sl)	-0.010	-0.10
			2	62.56		0.014		8.23				
		Bt/Cm/Sl	1	69.40	170.05	0.045	0.032	8.21	8.14	Sl-(Bt/Sl)	-0.014	-0.39
			2	270.70		0.019		8.08				
		Bt/Sl	1	60.25	62.73	0.035	0.035	8.61	8.43	Sl-(Cm/Sl)	-0.010	-0.44
			2	65.20		0.036		8.26				

		Cm/Sl	1	67.30	44.17	0.038	0.032	8.62	8.48	(Sl-(Bt/Sl))+(Sl-(Cm/Sl))	-0.024	-0.83
			2	21.03		0.025		8.34				
75 mM lactic acid [1.68 mM UDLA]	9	Sl	1	403.40	306.70	0.012	0.013	7.86	7.84	Sl-(Bt/Cm/Sl)	-0.002	-0.33
			2	210.00		0.015		7.82				
		Bt/Cm/Sl	1	316.40	253.35	0.018	0.015	8.31	8.17			
			2	190.30		0.013		8.04				
		Bt/Sl	ng[3]									
		Cm/Sl										
100 mM lactic acid [2.24 mM UDLA]	10	Sl	ng[3]									
		Bt/Cm/Sl										
		Bt/Sl	not tested									
		Cm/Sl										

[1] Bt, *Brochothrix thermosphacta*; Cm, *Carnobacterium malartomaticum*, Sl, *Serratia liquefaciens*

[2] equation used to calculate difference in MPD between 1-. 2-, and 3-species systems

[3] ng, no growth was observed

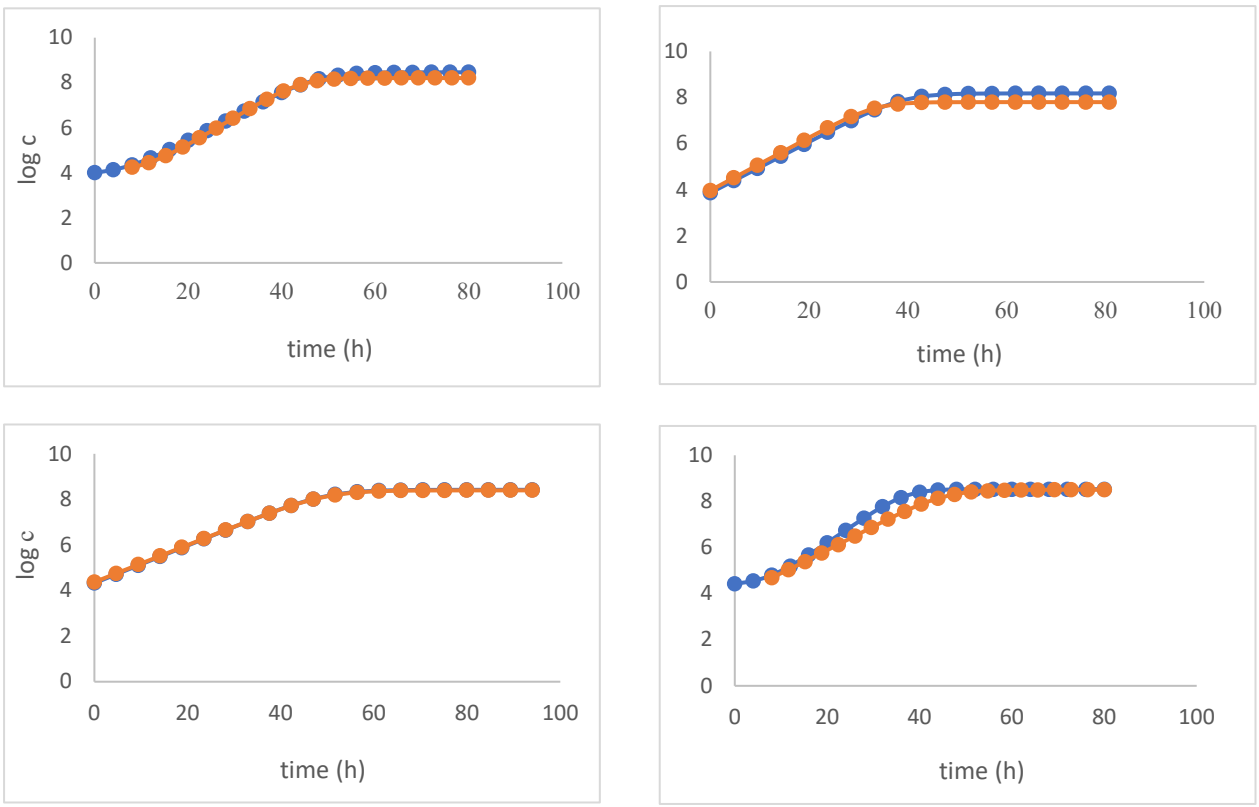


Figure C.1 *S. liquefaciens* grown in 0.00 mM (top panels) and 0.06 mM UDLA (bottom panels) at pH 6.5 as individual and 3-species mixed culture, for trials 1 (left panels) and 2 (right panels).

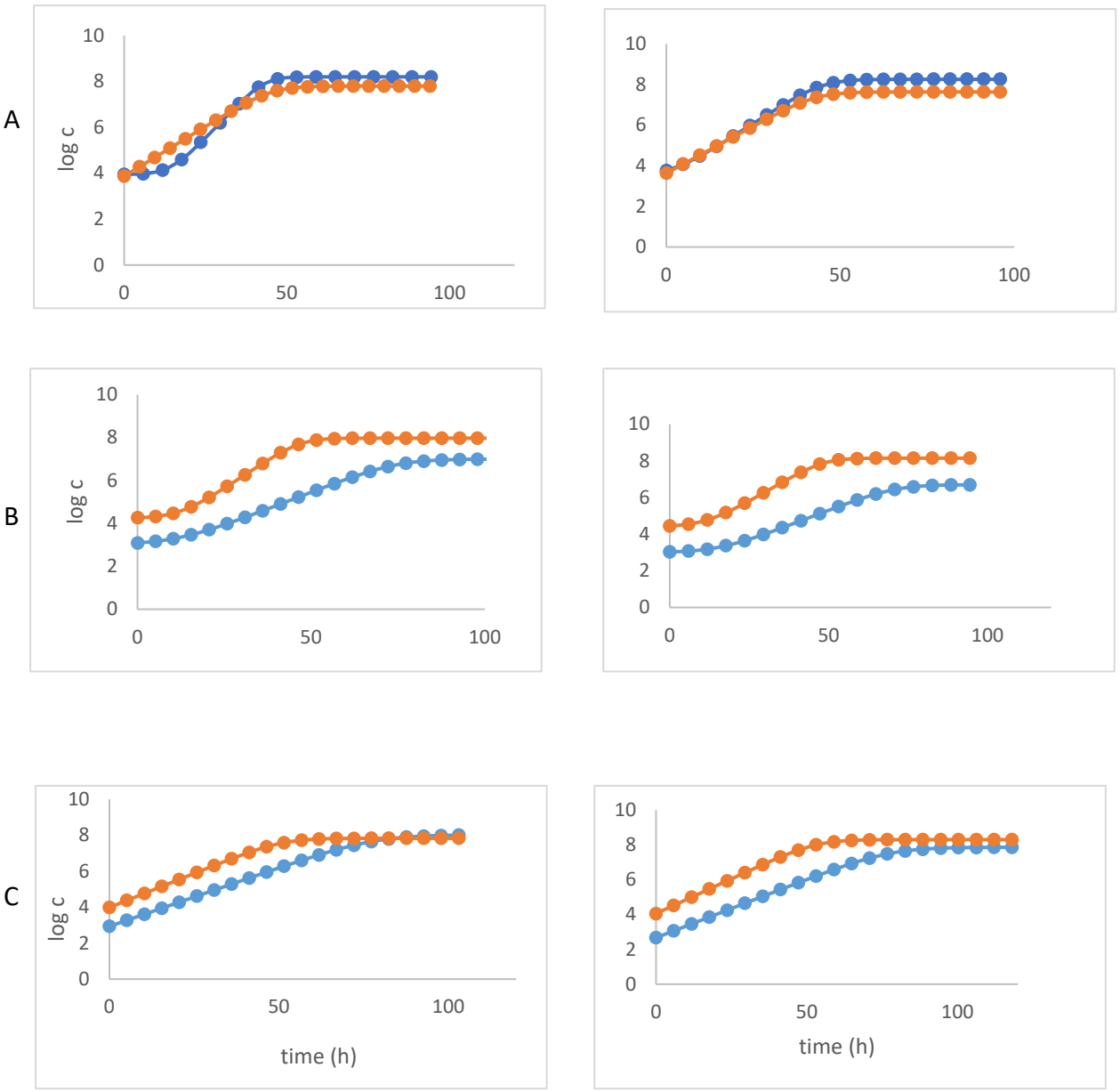


Figure C.2 (A) *S. liquefaciens* grown in 0.11 mM UDLA at pH 6.5 as single (blue line) and in 3-species culture (orange line), (B) *S. liquefaciens* (orange line) co-cultured with *B. thermosphacta* (blue line), and (C) *S. liquefaciens* (orange line) co-cultured with C*. maltaromaticum* (blue line), for trials 1 (left panels) and 2 (right panels).

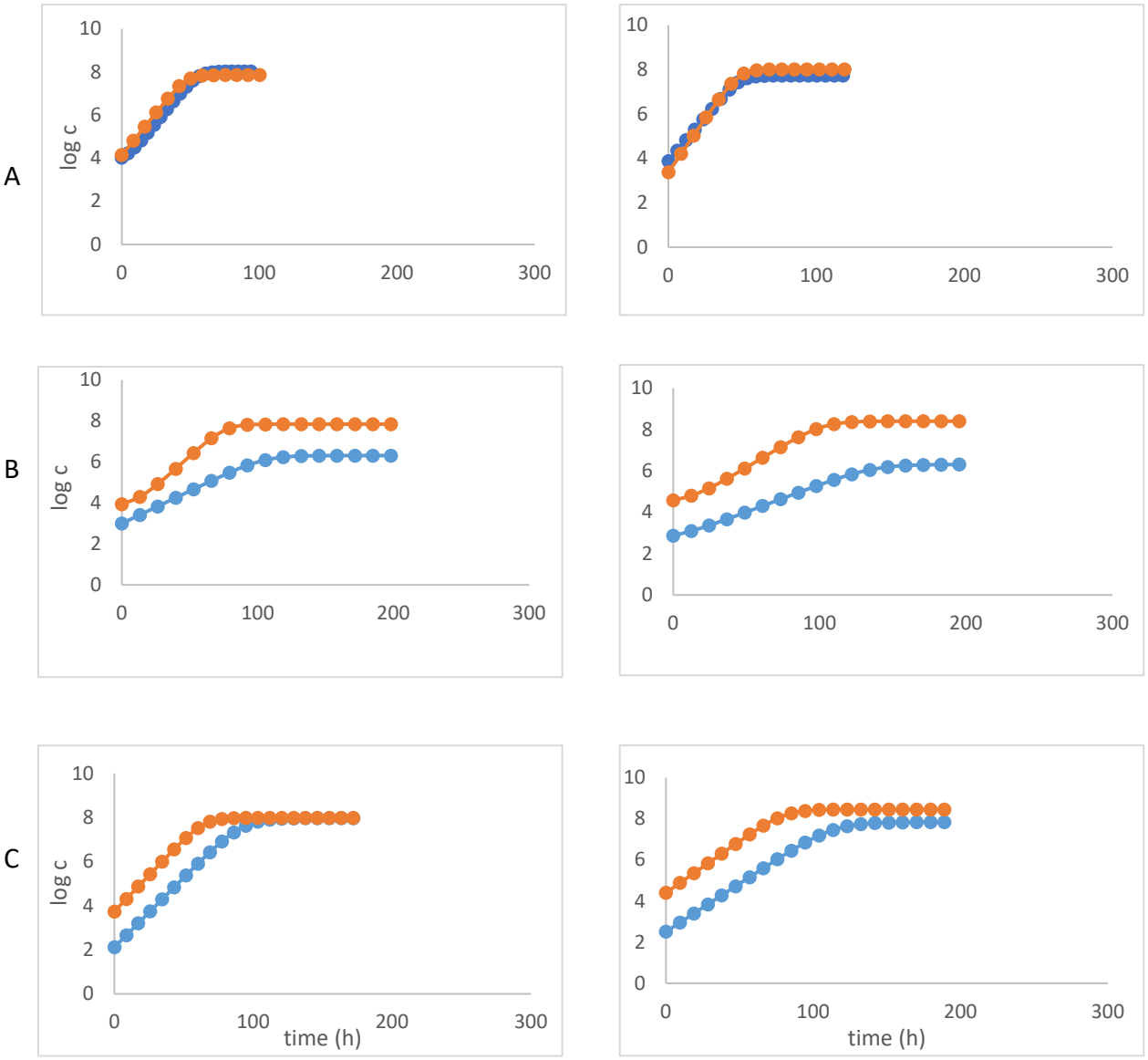


Figure C.3 (A) *S. liquefaciens* grown in 0.17 mM UDLA at pH 6.5 as single (blue line) and in 3-species culture (orange line), (B) *S. liquefaciens* (orange line) co-cultured with *B. thermosphacta* (blue line), and (C) *S. liquefaciens* (orange line) co-cultured with C*. maltaromaticum* (blue line), for trials 1 (left panels) and 2 (right panels).

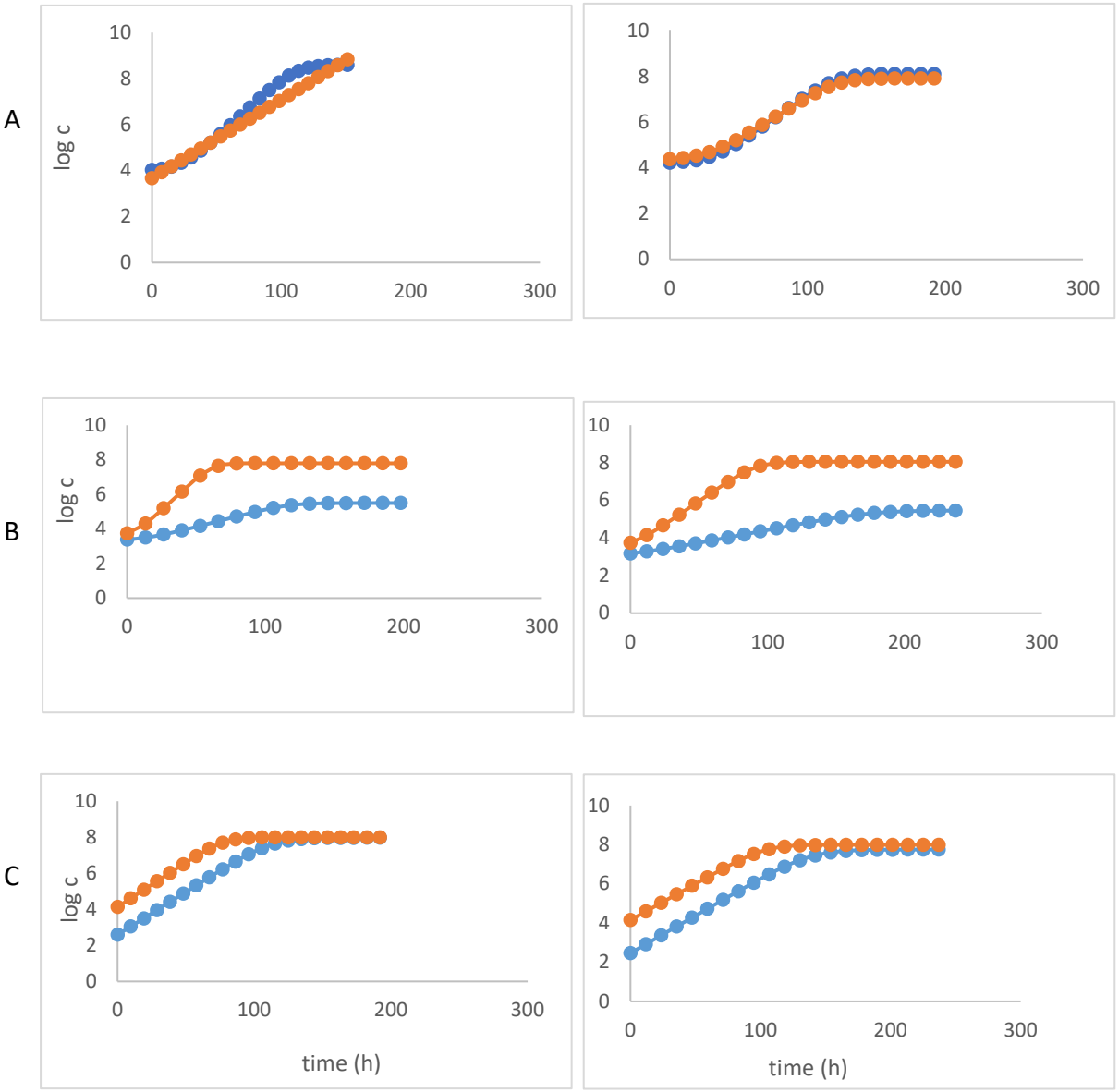


Figure C.4 (A) *S. liquefaciens* grown in 0.22 mM UDLA at pH 6.5 as single (blue line) and in 3-species culture (orange line), (B) *S. liquefaciens* (orange line) co-cultured with *B. thermosphacta* (blue line), and (C) *S. liquefaciens* (orange line) co-cultured with C*. maltaromaticum* (blue line), for trials 1 (left panels) and 2 (right panels).

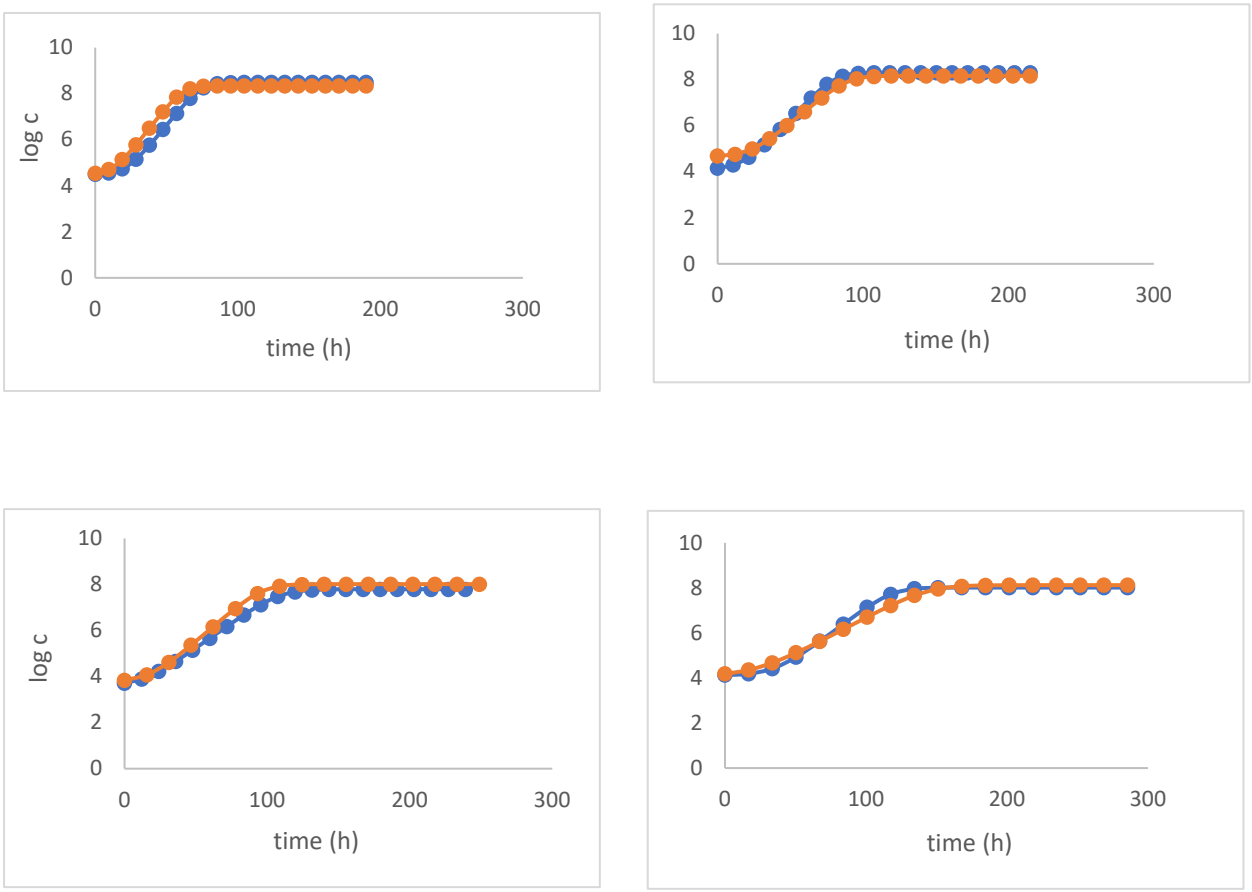


Figure C.5 *S. liquefaciens* grown in 0.00 mM (top panels) and 0.57 mM (bottom panels) at pH 5.5 as individual and 3-species mixed culture, for trials 1 (left panels) and 2 (right panels).

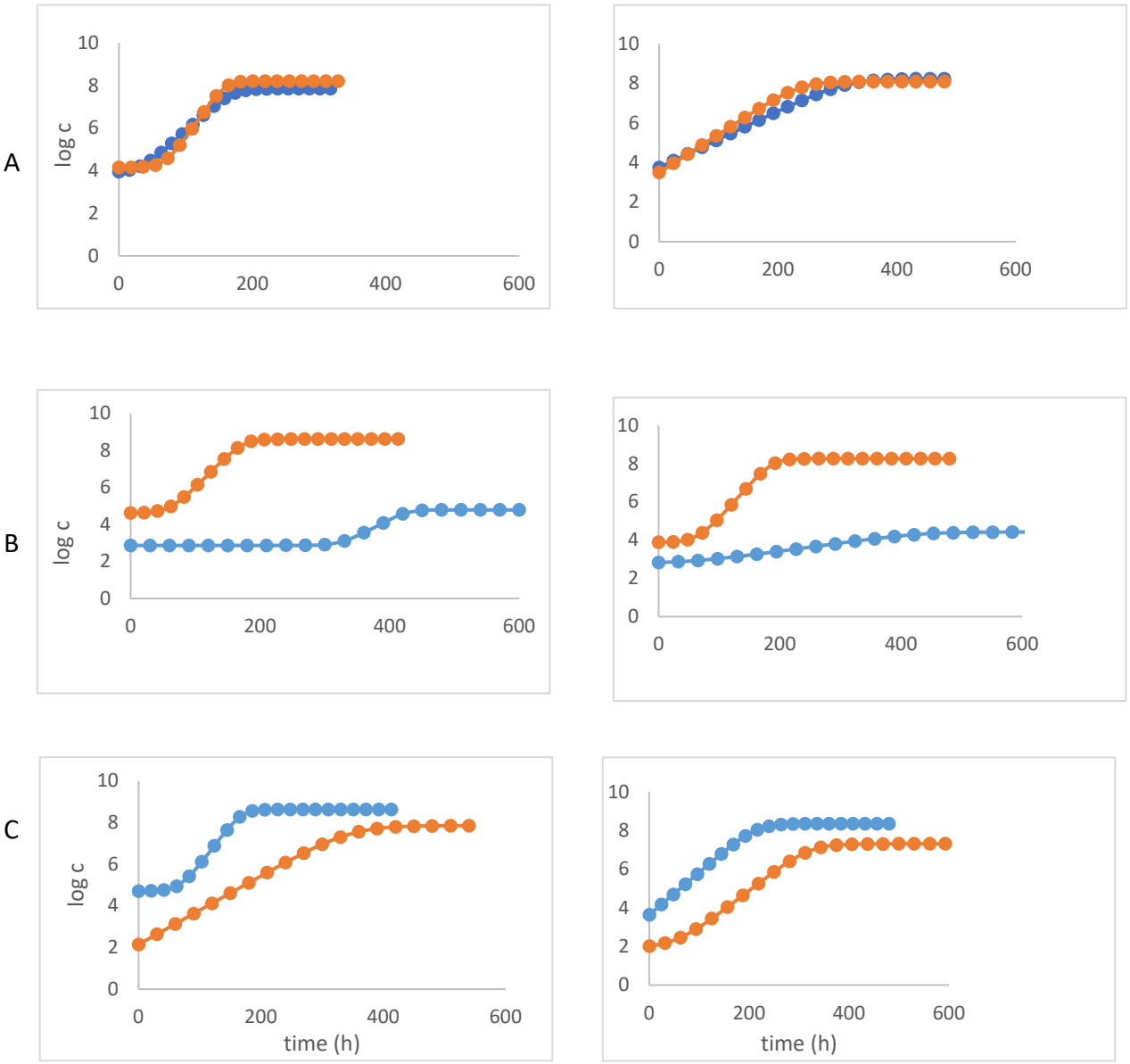


Figure C.6 (A) *S. liquefaciens* grown in 1.12 mM UDLA at pH 5.5 as single (blue line) and in 3-species culture (orange line), (B) *S. liquefaciens* (orange line) co-cultured with *B. thermosphacta* (blue line), and (C) *S. liquefaciens* (blue line) co-cultured with C. *maltaromaticum* (orange line), for trials 1 (left panels) and 2 (right panels).

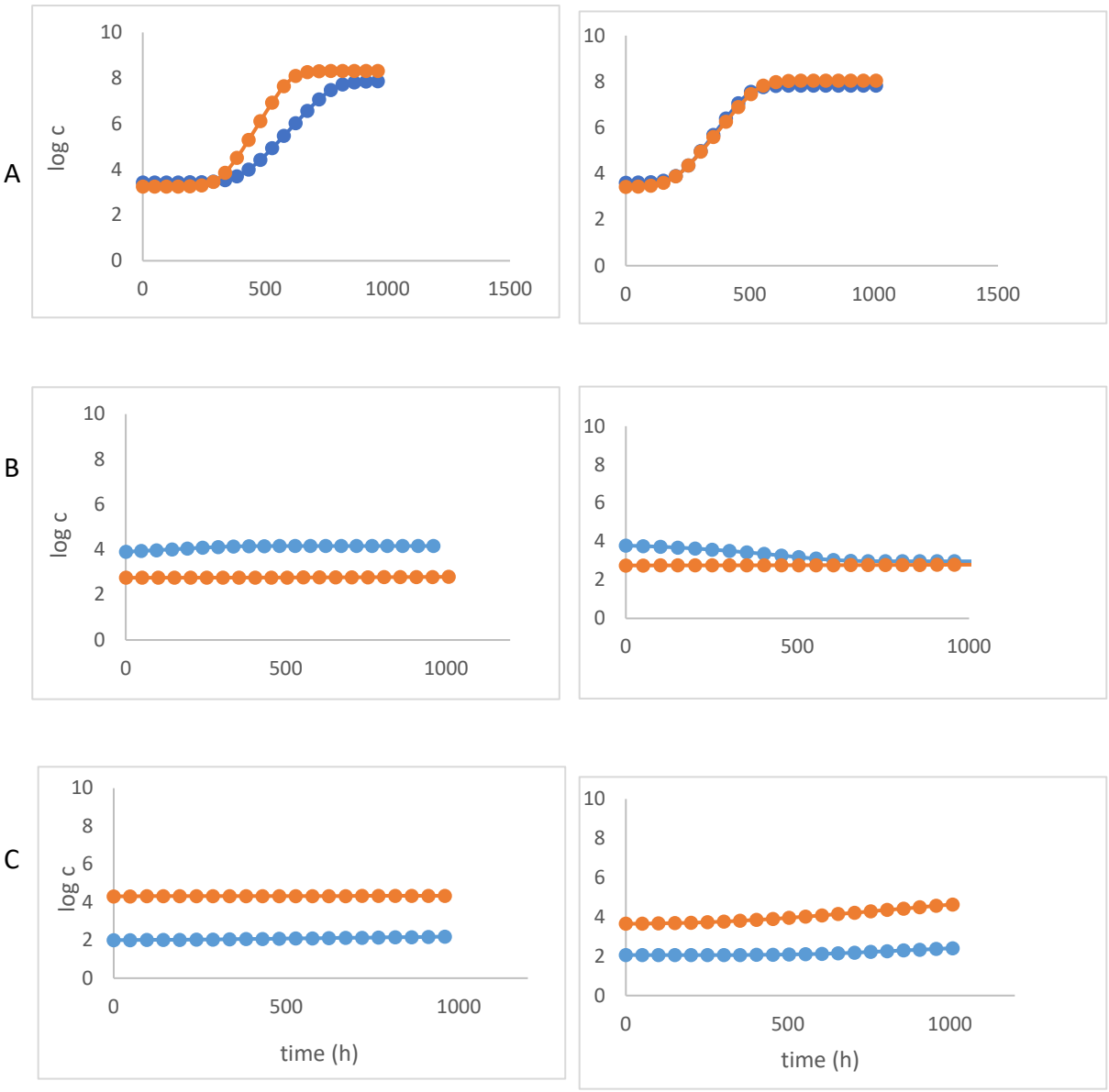


Figure C.7 (A) *S. liquefaciens* grown in 1.75 mM UDLA at pH 5.5 as single (blue line) and in 3-species culture (orange line), (B) *S. liquefaciens* (orange line) co-cultured with *B. thermosphacta* (blue line), and (C) *S. liquefaciens* (blue line) co-cultured with C. *maltaromaticum* (orange line), for trials 1 (left panels) and 2 (right panels).

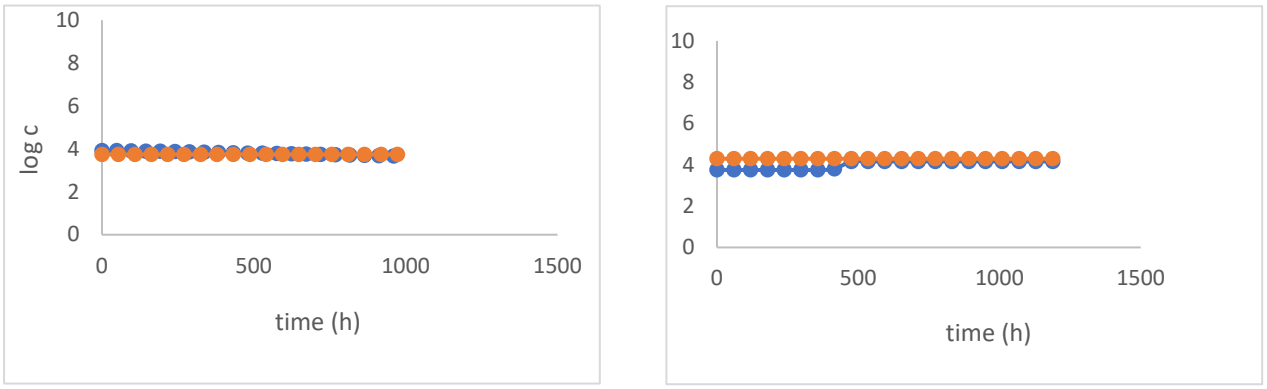


Figure C.8 *S. liquefaciens* grown in 2.24 mM UDLA at pH 5.5 as single (blue line) and in 3-species culture (orange line).

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