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# Interactions between bacterial strains isolated from vacuum-packaged Australian beef primals

by

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## Statement of co-authorship

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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## Publications arising from the thesis

### Journal publications

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- **Zhang P**, Kaur M, Bowman JP, Tamplin M. 2016. Effect of related environmental factors of vacuum-packaged beef on antibacterial compound production by *Carnobacterium maltaromaticum*. International Journal of Food Microbiology. To be submitted.

### Conference proceedings

- **Zhang, P**, Baranyi, J, Tamplin, M. 2014. 'Inter-strain interactions among bacteria isolated from Australian vacuum-packaged refrigerated beef', International Association for Food Safety 2014, European Symposium on Food Safety, Budapest, Hungary.
- **Zhang, P,** Ross T, Bowman, J, Williams, M, Tamplin, M. 2013. 'Inter-strain inhibitory activity within the bacterial community of Australian vacuum-packaged beef', University of Tasmania 2013 Graduate Research – Sharing Excellence in Research Conference, Hobart, Australia.
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## Table of contents

## Abstract

Vacuum-packaged (VP) beef produced and packaged in Australia can have an unusually long shelf-life. This observation has been attributed, in part, to superior abattoir hygiene, but there is an absence of robust scientific evidence to support this claim. While plant hygiene may be an important factor in extending VP beef shelf-life, there are likely other contributing factors.

Bacteria rarely exist in isolation and occur as members of a microbial niche. Numerous published studies have described the composition of bacterial species within food, including the influence of the environment; however, limited attention has been given to understanding how bacteria interact within foods, and how this contributes to the overall formation of a microbial community. The aim of the present study was to define how specific environmental factors, relevant to Australian VP beef, influence the interactions among bacteria.

Interactions among representative species of bacteria isolated from Australian VP beef primals were investigated. From a set of strains, 39 isolates inhibiting (effectors) other bacteria, and 20 isolates actively inhibited (targets) by effectors, were selected for further study. These isolates represented ten bacterial genera; including *Carnobacterium*, *Pseudomonas*, *Hafnia*, *Serratia*, *Yersinia*, *Rahnella*, *Brochothrix*, *Bacillus*, *Leuconostoc*, and *Staphylococcus*. A number of interactions were observed, with 28.6% inhibiting and 4.2% promoting target isolate growth. All lactic acid bacteria (LAB) inhibited other species, especially *Carnobacterium maltaromaticum,* which inhibited the growth of a wider range of target isolates, compared to other LAB. The majority of *Pseudomonas* isolates antagonised growth of approximately one-half of target isolates. Two *Bacillus* spp. each inhibited the growth of 16 target bacteria. The majority of effector isolates that enhanced target isolate growth were Gram-negative bacteria, including *Pseudomonas* spp. and *Enterobacteriaceae.*

The mechanisms of interactions were partially characterised for eight effector-target isolate combinations. The inhibitory effects of two isolates of *C. maltaromaticum* and one isolate of *Bacillus subtilis* was mediated by heat-stable, pH-stable, proteinaceous substances found in cell-free supernatants (CFS). In contrast, live cells were required for the inhibitory activity of three isolates of *Bacillus* sp., *Pseudomonas putida,* and a *Pseudomonas* sp. against corresponding isolates of *Yersinia enterocolitica, C. maltaromaticum* and *B. subtili*s, yet this inhibitory effect did not require direct contact between effector and target cells. Compounds produced by *B. subtilis* and *Serratia* sp. that promoted the growth of *Pseudomonas lundensis* were non-proteinaceous and were heat- and pH-stable.

The next phases of the thesis quantified the effect of simulated intrinsic VP beef factors and associated extrinsic storage conditions (i.e. pH, temperature, atmosphere, glucose, and lactic acid) on: 1) the sensitivity of target isolate *C. maltaromaticum* strain D8c to inhibition caused by effector strain *C. maltaromaticum* D0h and, 2) the production of inhibitory factor(s) by *C. maltaromaticum* D0h. In the former study, all five environmental factors significantly ($P < 0.05$) affected sensitivity of *C. maltaromaticum* D8c to D0h CFS inhibitory activity. Inhibition sensitivity was relatively higher at low pH (5.5), at higher concentrations of glucose (5.55 mM) and lactic acid (50 mM), and under aerobic conditions. The sensitivity of strain D8c did not correlate linearly with temperature; since inhibition was greatest at 15°C, followed by 7, -1, and 25°C. Preliminary models were produced to describe D8c sensitivity.

Furthermore, the influence of pH, atmosphere, glucose, and lactic acid on production of inhibitory compounds by D0h was studied at 25°C. It was found pH produced the greatest influence on inhibitor production, compared to atmosphere, glucose, and lactic acid. The lowest amount of inhibitor was produced at an initial medium pH of 5.5. Lactic acid significantly reduced production, but only at an initial pH of 5.5. A two-factor interaction was observed between glucose and pH; relatively high concentrations of glucose (5.55 mM)

enhanced the production at pH 6.5, whereas production decreased at pH 5.5. Atmosphere did not significantly affect inhibitory activity.

In conclusion, numerous interactions among the bacterial community of VP beef were described, and potentially drive formation of the microbial spoilage community, as influenced by environment. Intraspecific interactions between two *C. maltaromaticum* isolates were significantly affected by pH, atmosphere, lactic acid, glucose, and temperature. These findings, and the resulting models, may improve the understanding of putative interactions among spoilage bacteria in meat, in particular *C. maltaromaticum*, one of the most dominant bacterial species on chilled VP beef.

# Chapter 1

# General introduction

## BACKGROUND AND STUDY AIMS

Australia is one of the largest global exporters of beef, shipping beef products to more than 100 countries. Export has shown sustained growth over the past ten years and reached 1.29 million tonnes in 2014, accounting for 74% of the total Australian beef production (MLA, 2015). Consequently, producing high-quality beef is a primary goal of the Australian meat industry.

Long beef shelf-life is a prerequisite, especially for international markets compared to domestic market. Beef products for exports are normally vacuum-packaged (VP) as primal cuts in plastic films (CSIRO, 2009). Australian VP beef primals stored at optimal commercial conditions (-0.5$^{o}$C) have a long shelf-life, 26–30 weeks (Small et al., 2012), however, the scientific reason for this is not well elucidated. Besides low levels of bacteria present before packaging, a specific bacterial composition, and interactions among bacteria, may contribute to the enhanced shelf-life.

Bacterial interactions are one of the underlying forces that influence diversity of bacterial community in a niche (Blana and Nychas, 2014; Faust and Raes, 2012; Perez-Gutierrez et al., 2013; Wolfe et al., 2014). Bacteria compete via secreting defensive compounds, directly 'scrambling' for nutrients, or performing contact-dependent inhibition (Avendano-Perez and Pin, 2013; Cotter et al., 2013; Faust and Raes, 2012; Hayes et al., 2014; Hibbing et al., 2010; Oliveira et al., 2008; Russell et al., 2011; Vaughan et al., 2001). Conversely, some bacteria are able to cooperate by exchanging metabolic products or via quorum sensing systems

(Buckling et al., 2007; Faust and Raes, 2012; Giaouris et al., 2015; Pande et al., 2015; Ponomarova and Patil, 2015; Skandamis and Nychas, 2012). These interactions may also be affected by environmental factors, for example, pH, temperature, and available nutrients. However, research leading to understanding these processes, as they pertain to food-sourced bacteria, remains limited.

Therefore, this thesis aims to elucidate:

a. interactions among bacterial genera that predominate on VP beef;
b. possible mechanisms mediating relatively strong interactions between VP beef-associated bacteria;
c. effects of environmental factors on these interactions.

## STRUCTURE OF THE THESIS

This thesis contains seven chapters:

Chapter 1 (this chapter): The background, study aims, and the structure of the thesis are described.

Chapter 2: The background research regarding potential contamination sources of VP beef during production, dominant bacterial species on VP beef, and putative factors in bacterial interactions are reviewed.

Chapter 3: The interactions among bacterial isolates of ten genera obtained from Australian VP beef, including *Carnobacterium*, *Leuconostoc*, *Brochothrix*, *Pseudomonas*, *Serratia*, *Hafnia*, *Rahnella*, *Yersinia*, *Bacillus*, and *Staphylococcus*, are described.

Chapter 4: The possible mechanisms mediating the interactions found in the previous chapter, to help understand the effect of environmental factors on these interactions, are elucidated. Eight combinations of effector and target isolates showing relatively strong interactions were studied.

Chapter 5: The effects of environmental factors of VP beef on the sensitivity of target isolate *Carnobacterium maltaromaticum* D8c to inhibitory compounds produced by effector isolate, *C. maltaromaticum* D0h, are described. This chapter has been submitted to a refereed journal, and includes preliminary characterization of interacting compounds produced by *C. maltaromaticum* D0h, data for which are also shown in Chapter 4.

Chapter 6: The effects of environmental factors on production of inhibitory factors by effector isolate *C. maltaromaticum* D0h are explained.

Chapter 7: The results achieved in this thesis and future research directions are discussed.

# Chapter 2

# Literature review

Australian vacuum-packaged (VP) beef primals have a long shelf-life. Small et al. (2012) investigated striploins and cube rolls collected from six Australian processing plants located over a wide range of latitudes, ranging from Tasmania (41.5°S) to northern Queensland (19.2°S). The study proved that beef primals achieved a shelf-life of at least 26 weeks, and bacterial counts on beef sample surface rarely reached 7 log cfu/cm$^2$ even after 30 weeks. Well-managed beef processing (low initial microbial loads at the time of packaging) and storage (low temperature and low oxygen gaseous atmosphere) conditions may together contribute to the extended shelf-life. The composition of the meat bacterial community and bacterial interactions may also benefit extended long VP beef shelf-lives (Youssef et al., 2014a).

This review first introduces the process of beef production and potential contamination sources during production, the microbial community associated with VP beef spoilage, and environmental factors influencing spoilage. Thereafter, the review progresses to discussing bacterial interactions, possible influential environmental factors, and predictive models that consider bacterial interactions.

## BEEF PRODUCTION AND POTENTIAL CONTAMINATION SOURCES

Livestock are prepared to be slaughtered once arriving at an abattoir. Pre-slaughter washing is normally applied to cattle to clean their hides (Byrne et al., 2000). Cattle are

immediately bled after stunning, followed by carcass processing in a series of procedures including skinning, evisceration, trimming and carcass washing, weighing, grading, and chilling (Rowlands, 2010). Beef carcasses are then chilled to 4$^{o}$C or lower within 12 hours after slaughter (Rowlands, 2010).

Initial microbial loads play a significant role in determining the quality of meat; as a result an hygienic environment is required during meat production (CSIRO, 2009). In general, the muscle tissue of a healthy animal is free of microorganisms, while the surface of the carcass is frequently contaminated during slaughter and processing (Huffman, 2002; Kperegbeyi, 2014). Significant differences in beef carcass contamination have been found among animal farms, abattoirs and meat processing plants (Sofos et al., 1999; Zweifel et al., 2005).

The processing sites for hide removal are the first immediate sources of carcass contamination (Bell, 1997). Contamination from within the processing environment (e.g. hands of workers) and from animal hides may be transferred to the surface of carcasses (Aslam et al., 2003; McEvoy et al., 2000). A positive relationship between the extent of hide contamination and bacterial loads on cattle carcasses has been found (McEvoy et al., 2000). Animal hides, especially the areas of the brisket, distal leg, and crotch, are the most easily contaminated by microorganisms from soil and faeces (Antic et al., 2010; Reid et al., 2002). Nevertheless, information on the diversity of bacterial species that may contaminate carcasses and subsequently affect shelf-life of beef products remains limited. Material from the gastrointestinal tract is an another contamination source, especially during evisceration if perforation occurs (CSIRO, 2009). Post-evisceration washes are able to reduce the bacterial counts on carcasses and also lead to the redistribution of organisms on carcasses (Bacon et al., 2000; Bell, 1997). Bacterial contaminants can include a diverse range of bacteria, for example, *Clostridium*, *Acinetobacter*, and *Enterococcus* (Callaway et al., 2010; Dowd et al., 2008).

After carcass chilling the next step is boning, in which carcasses are cut into small pieces or primals, for example, silverside, blade, striploin, and cube roll (AUS-MEAT, 2010). Packaging is often finished within 24 hours from slaughter. For Australian beef, primal cuts (2–9 kg) are normally vacuum-packaged in plastic film before being sold internationally or to domestic retailers (CSIRO, 2009). To ensure a longer storage life, packaging films should have low permeability to gases. In general, a small fraction of oxygen remains in the package and is absorbed by muscle tissue and microorganisms that exist on the meat surface. The storage temperature is typically $-0.5 \pm 0.5^{o}C$ under optimal commercial conditions, especially for export, and is a key factor influencing the shelf-life of beef products (CSIRO, 2009; Small et al., 2012).

## VP BEEF SPOILAGE ASSOCIATED BACTERIA

The spoilage of meat is a deterioration process of its sensory quality, which is caused by protein degradation of meat itself, and more importantly by the metabolic activity of its microbial community (Borch et al., 1996; Ellis et al., 2002; Nychas and Tassou, 1997). The development of off-odours, off-flavours, discoloration, and slime formation renders meat products unacceptable for human consumption. During meat processing and packaging, microorganisms have opportunities to attach to meat surface via van der Waals' or electrostatic forces, and then steadily colonize it via glycocalyx formation (Chung et al., 1989; Zulfakar et al., 2012). Microorganisms are able to consume meat nutrients such as glucose, amino acids, and lactic acid, and produce undesirable metabolites including alcohols, ketones, amines, sulphur compounds, and organic acids. Proteins are often used as carbon sources by spoilage microbes at the end of meat storage (Montel et al., 1998; Nychas et al., 2008). Lactic acid bacteria (LAB), *Enterobacteriaceae*, *Pseudomonas*, *Clostridium*, and *Brochothrix thermosphacta* are the most common types of bacteria involved in beef spoilage (Brightwell

et al., 2009; Doulgeraki, 2010; Doulgeraki et al., 2012; Ercolini et al., 2009; Ferrocino, 2009; Pennacchia et al., 2011).

**Lactic acid bacteria.** Psychrotrophic LAB are the most common bacteria associated with chilled VP beef spoilage (Borch et al., 1996). At the beginning of storage, LAB are often below detection limits; however, due to their adaptive capacity under anaerobic refrigeration conditions, their populations gradually increase and dominate the microbial community when maximum spoilage populations are reached (Hernandez-Macedo et al., 2011); the mechanisms by which they eventually predominate are not well described.

*Lactobacillus*, *Leuconostoc*, *Enterococcus*, *Lactococcus*, and *Carnobacterium* are the main LAB genera that are well adapted in VP beef environments (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). In the study of Jones (2004), *Enterococcus gilvus* was reported as the most dominant LAB on chilled VP beef samples followed by *Carnobacterium divergens*, *Enterococcus faecium*, and *Leuconostoc mesenteroides*. However, Sakala et al. (2002) reported *Leuconostoc gelidum*, *Lactococcus piscium* and *Lactobacillus algidus* as dominant LAB species on VP beef stored at 2$^{o}$C for up to 6 weeks, and *C. divergens* and *C. maltaromaticum* were present at relatively low levels. Brightwell et al. (2009) reported members of the genus *Carnobacterium* as the most abundant LAB in spoilage communities on VP New Zealand beef with *C. divergens* and *C. maltaromaticum* the two main species present.

The dominant LAB species composition on VP beef can be affected by contamination during processing, storage temperature, and the vacuum-packaging method (traditional vacuum-package (TVP) versus advanced vacuum skin packaging (AVSP)) (Samuel et al., 2011; Yang et al., 2009). *Carnobacterium maltaromaticum* displayed a higher growth rate at

0–4$^{o}$C compared to *L. mesenteroides* and *Lactobacillus lactis*; whereas, the growth rates of these species were similar at 4–8$^{o}$C (Yang et al., 2009). Samuel et al. (2011) applied a high temperature heating of package film immediately before it attached onto beef surface (AVSP); a lower proportion of *L. mesenteroides* was found compared to TVP without heating.

A few LAB species contribute to meat spoilage through released metabolites, leading to organoleptic deterioration of meat (Pothakos et al., 2015). LAB are able to utilize glucose and other substrates in meat, and then produce metabolites having acidic or milky flavours, and cheesy or dairy odours. Occasionally, LAB may also produce gas ($CO_2$) that can lead to package failure (Hanna et al., 1979; Hernandez-Macedo et al., 2011; Jones, 2004). On beef steaks, *Leuconostoc gasicomitatum* can cause green discolouration and off-odours (Vihavainen and Bjorkroth, 2007). This species can produce diacetyl, which manifests as buttery off-odours of meat products (Jaaskelainen et al., 2013; Johansson et al., 2011; Pothakos et al., 2014). Growth of *L. mesenteroides* can account for increased levels of butyric acid in meats, a compound associated with rancid/buttery flavours and odours (Jones, 2004). *Carnobacterium maltaromaticum* and *L. sakei* have also been reported to cause off-odours and discoloration of VP beef slices stored at 2$^{o}$C (Leisner et al., 1995). *Carnobacterium maltaromaticum* produces volatile compounds, for instance, acetoin, 1-octen-3-ol, butanoic acid, aldehydes, lactones, and sulphur-containing compounds, all of which are related to beef deterioration (Casaburi et al., 2011; Ercolini et al., 2009). However, Casaburi et al. (2011), who investigated the spoilage potential of 54 strains of *C. maltaromaticum*, considered that the overall impact of *C. maltaromaticum* metabolizing activity on VP meat spoilage is either weak or negligible.

On the contrary, LAB have been regarded as potential protective cultures due to their ability to produce organic acids and/or bacteriocins, which are unfavourable to the growth of other bacteria (Pothakos et al., 2015; Signorini et al., 2006). *Lactobacillus curvatus* CRL705

extended the shelf-life of chilled VP beef and delayed beef tissue degradation for 10 days (Castellano et al., 2010). Also, blown-pack spoilage of VP beef was delayed by *Lactobacillus sakei* (Jones et al., 2009). In the study of Katikou et al. (2005), *L. sakei* 4808 reduced the spoilage microbial counts of VP sliced beef, while *L. curvatus* CECT $904^T$ did not have a significant effect.

Overall, the role of LAB in meat spoilage is ambiguous, which is probably due to the heterogeneity observed amongst this group of bacteria (Pothakos et al., 2015). Future studies are still needed to understand the potential role of LAB in meat shelf-life at species or even strain level.

***Brochotrix thermosphacta.*** *Brochthrix thermosphacta* is a Gram-positive, non-spore forming, homofermentative, facultatively anaerobic rod-shaped bacterium. This species was first described from pork sausages in 1951 (Casaburi et al., 2014; Nowak et al., 2012; Sulzbacher and Mclean, 1951). Since then, it has been frequently detected on lamb, beef, fish, and cured meats (De Filippis et al., 2013). In the investigation by Nowak et al. (2012), *B. thermosphacta* was only absent in 2 of 132 meat samples.

The development of *B. thermosphacta* in meat products displays a negative impact on meat sensory properties (Braun and Sutherland, 2003; McClure et al., 1993). It has the potential to produce histamine, putrescine, tyramine, cadaverine and tryptamine in meats stored aerobically (Emborg et al., 2005; Nowak and Czyzowska, 2011; Papadopoulou et al., 2012). Proteolytic and lipolytic activities, which are associated with organoleptic changes of meats, have been reported for bacteria of this species, but variability exists among strains. For instance, in the study of Casaburi et al. (2014), none of the strains was able to produce lipase or digest proteins *in vitro* or *in situ* at $4^oC$ or $20^oC$. Conversely, some other authors reported lipolytic activity of various *B. thermosphacta* strains and found that most strains prefer

relatively high temperatures for synthesizing lipase, for example, 20$^{o}$C in the studies of Papon and Talon (1988) and Braun and Sutherland (2003), and 25$^{o}$C in the study of Nowak et al. (2012). The strain studied by Labadie (1999) did not degrade proteins, while Braun and Sutherland (2003) observed proteolytic enzymes produced by *B. thermosphacta* in stationary growth phase but did not observe exoproteases at temperatures below 6$^{o}$C. According to these studies, temperature is an important factor influencing lipolytic or proteolytic activity; however, the effect of oxygen remains unclear.

*Brochotrix thermosphacta* is able to grow at refrigerated temperatures under anaerobic conditions (Pin et al., 2002). However, its growth capability in the absence of oxygen is relatively weak than when oxygen is present (Kamenik et al., 2014; Pennacchia et al., 2011; Sakala et al., 2002). Studies showed that vacuum-packaging reduced the viable counts of *B. thermosphacta*, and this species is not able to compete against LAB in VP chilled meats (Crowley et al., 2010; Gribble and Brightwell, 2013; Pennacchia et al., 2011; Russo et al., 2006; Sakala et al., 2002). The environmental factors in VP meat, such as lactic acid, may also inhibit the growth of *B. thermosphacta* (Bell et al., 2001; Grau, 1980; Newton et al., 1978). In the study of Bell et al. (2001), *B. thermosphacta* failed to grow on VP beef possibly due to relatively high levels of lactic acid in the meat tissue.

The presence of oxygen not only affects the growth of *B. thermosphacta* but also its carbohydrate metabolism (Pin et al., 2002). Under anaerobic conditions, it mainly causes off-odours by producing L-(+)-lactic acid, ethanol, and small amounts of short chain fatty acids via consumption of glucose; under aerobic conditions, acetoin, which is considered to be related to creamy dairy odour of meat, has been used as a spoilage marker of saveloy, and is the most important sensory-related compound produced by *B. thermosphacta*, (Casaburi et al., 2014; Casaburi et al., 2011; Holm et al., 2013; Holm et al., 2012).

***Enterobacteriaceae.*** Members of the family *Enterobacteriaceae* are often present amongst the spoilage flora on VP beef (Sakala et al., 2002; Youssef et al., 2014a; Youssef et al., 2014b). Fresh beef normally contains a small population of enterobacteria, and the number increases during storage (Degirmencioglu et al., 2012). Due to lack of oxygen, vacuum-packaging generally reduces the number of enterobacteria on beef compared to aerobic packaging (Pennacchia et al., 2011); therefore their populations are normally lower than that of LAB (Chaves et al., 2012; Degirmencioglu et al., 2012; Youssef et al., 2014b).

*Serratia* spp., *Hafnia alvei*, *Rahnella* spp., and *Yersinia enterocolitica* are the most frequently encountered taxa detected on VP 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). *Hafnia* and *Serratia* have been reported to produce diamine and cause spoilage of meat (Dainty et al., 1986; Edwards et al., 1985; Gill and Penney, 1988; Nortje and Shaw, 1989). Members of *Enterobacteriaceae*, especially *H. alvei* have the potential to cause "blown pack" spoilage, which is characterized as gas production and pack distension (Brightwell et al., 2007; Chaves et al., 2012; Hanna et al., 1979; Hernandez-Macedo et al., 2011; Kang et al., 2002). Ercolini et al. (2009) reported that *Serratia proteamaculans* produced high concentration of alcohols including 1-octen-3-ol, and esters, such as isoamyl acetate.

***Pseudomonas.*** Pseudomonads are also major contaminants of meat and are often isolated from fresh beef (Chandran et al., 1986; De Filippis et al., 2013; Doulgeraki et al., 2012; Labadie, 1999; Nychas et al., 2008; Sakala et al., 2002; Venter et al., 2006). They are regarded as important spoilers of meat stored aerobically (Borch et al., 1996; Doulgeraki et al., 2012). On VP beef, the growth of *Pseudomonas* spp. is normally restricted by limited available oxygen (Pennacchia et al., 2011). However, *Pseudomonas* spp. have been reported to grow under vacuum-packaging, which may be due to the relatively high gaseous

permeability of vacuum films (Ercolini et al., 2010; Newton and Rigg, 1979; Tsigarida and Nychas, 2001). In the study of Pennacchia et al. (2011), *Pseudomonas* spp. were detected in all stages of VP beef sample storage. *Pseudomonas fragi* is one of the most prevalent species contaminating meats and has the potential to produce 1-octen-3-ol, an off-odour of meat (Ercolini et al., 2011; Ercolini et al., 2009). In addition, high amounts of alcohols and ketones were detected in the head space of VP beef samples inoculated with *P. fragi* (Ercolini et al., 2009). *Pseudomonas 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).

***Clostridium.*** In some instances, blown-pack spoilage (BPS) may unpredictably occur in chilled VP meats including beef, pork and poultry, at an early stage of storage (Adam et al., 2010). Due to a large amount of gas production, primarily $CO_2$, package distention ("blown") often happens to BPS meats (Hernandez-Macedo et al., 2012). Psychrotrophic clostridia including *Clostridium algidicarnis, C. algidixylanolyticum, C. estertheticum, C. frigidicarnis, C. gasigenes*, and *C. putrefaciens* have been regarded as the main causative microorganisms of blown-pack spoilage of VP chilled meat that results in the production of abundant gas, off-odours, exudates, proteolysis and changes in pH and colour (Adam et al., 2011; Adam et al., 2010; Broda et al., 2002; Caplice and Fitzgerald, 1999; Cavill et al., 2011; Silva et al., 2011). In a four-year survey of the incidence of *C. estertheticum* and *C. gasigenes*, primal cuts in Ireland were found to be contaminated by these bacterial species at a low but persistent level (0.2–4.3 %) (Bolton et al., 2015).

**Other species**. Fresh beef products often also contain other bacterial species besides the main contaminants mentioned above (Youssef et al., 2014b). However, due to the selection pressure of subsequent storage conditions, it is difficult for these species to dominate the

microbial community on VP beef (Ercolini et al., 2011). Species of the genera *Acinetobacter*, *Staphylococcus*, *Shewanella*, and *Bacillus* have been detected during the early stages of VP beef storage (Doulgeraki et al., 2012; Ercolini et al., 2009; Sakala et al., 2002; Youssef et al., 2014b). *Shewanella putrefaciens* is able to utilize cysteine of meat and produce hydrogen sulphide and organic sulphides, which have unpleasant odours and react with myoglobin to cause greening of meat (Hernandez-Macedo et al., 2011). *Microbacterium*, *Flavobacterium*, *Moraxella*, *Ralstonia*, *Limnobacter*, and *Photobacerium* can also occur on beef (Doulgeraki et al., 2012; Ercolini et al., 2011; Pennacchia et al., 2011; Youssef et al., 2014b). Argyri et al. (2011) also reported minced beef samples contained yeasts and moulds.

## POTENTIAL FACTORS INFLUENCING VP BEEF SPOILAGE

**Initial microbial population.** The initial population of microorganisms contaminating beef strongly influences shelf-life (Yang et al., 2014b); hence, keeping hygienic production conditions is crucial. The animal hide is well recognised as a main source of carcass contaminants. Various carcass decontamination treatments have been applied in abattoirs, such as washing with hot water and organic acids (e.g. lactic acid), irradiation and steam pasteurization (Baird et al., 2006; Edwards and Fung, 2006). Investigations have shown that hot water, lactic acid, sodium hydroxide and phosphoric acid reduce the cell counts of *Enterobacteriaceae* (Baird et al., 2006; Bosilevac et al., 2005). Greig et al. (2012) analysed 202 experimental trials in the published literature and applied a systematic meta-analysis. They concluded that some carcass treatments, hot water washing, steam pasteurization and dry-chilling, are effective measures in reducing generic and pathogenic strains of *E. coli* contamination on beef carcasses. Hydrodynamic pressure processing applied before beef

packaging was also reported to be efficient in reducing initial cell density (Williams-Campbell and Solomon, 2002).

**Packaging films.** Vacuum-packaging protects beef from contact with oxygen, thus inhibiting the growth of aerobic bacteria, such as *Pseudomonas* spp. (Hernandez-Macedo et al., 2011). The low permeability of VP film to oxygen is important to ensure a long shelf-life of beef. However, in the beginning of VP beef storage, the residual oxygen or dissolved oxygen existing inside the package may allow slight growth of aerobic bacteria. Barros-Velazquez et al. (2003) improved the vacuum-packaging system by introducing instantaneous heating of the packaging film before it tightly attached to the meat surface; beef cuts with this advanced vacuum skin system displayed an overall higher quality. Post-packaging heat shrinking was reported to accelerate the onset of blown pack spoilage mediated by *Clostridium* spp. (Bell et al., 2001). Conversely, beef spoilage was retarded by the combination of vacuum pressure (9 mbar) and shrinking temperature (87$^{o}$C) according to Silva et al. (2012).

**Use of antimicrobial agents along with vacuum-packaging.** To extend beef shelf-life, beef processors may add antimicrobial agents to meat being vacuum-packed. Lactic acid application on the surface of beef, vacuum-packaging, and storage at 4 and 20$^{o}$C, proved to be efficient for controlling spoilage populations including *Enterobacteriaceae*, *Pseudomonas* spp., and *B. thermosphacta* (Signorini et al., 2006). Other chemicals including sodium lactate and peroxyacetic acid also have the potential to extend VP beef shelf-lives (Brightwell et al., 2009; Maca et al., 1997). Bacteriocins, as optimal alternatives to antibiotics, have also been applied to control spoilage bacteria on VP beef. Nisin was found to be efficient in reducing the cell counts of *B. thermosphacta* on VP beef (Siragusa et al., 1999; Tu and Mustapha, 2002). Application of protective cultures is another option to retard meat spoilage. According to Castellano et al. (2010), application of *Lactobacillus curvatus* CRL705 as a protective

culture retarded VP beef deterioration for 10 days. Certain strains of *L. sakei* have shown some potential to improve VP beef shelf-life (Jones et al., 2009; Katikou et al., 2005). In addition, Aksu et al. (2015) applied aqueous extracts of lyophilized *Urtica dioica* L. (stinging nettle), which includes natural plant antioxidants, in VP beef steaks and found that this substance delayed beef spoilage for >3 days at 450 ppm and for 3 days at 150–300 ppm, respectively; the growth of LAB, *Pseudomonas,* and *Enterobacteriaceae* were all decreased.

**pH.** pH is an important intrinsic environmental factor that influences the growth of bacteria. In general, the pH of raw beef is approximately 5.5, subject to the effects of animal feeding and handling, and initial intervention treatments used on cattle carcasses (Alasvand Zarasvand et al., 2012; Argyri et al., 2011; Blixt and Borch, 2002). The pH of beef stored anaerobically usually decreases during storage (Irkin et al., 2011). LAB, which can produce lactic acid, is a major factor contributing to pH decrease of VP beef (Irkin et al., 2011).

Some studies indicated that the growth of pseudomonads was inhibited by low pH (Blixt and Borch, 2002; Koutsoumanis et al., 2006), while in other research the growth kinetics of *Pseudomonas* did not change with the change in pH range from 5.3–7.8 (McMeekin and Ross, 1996). Some researchers argue that a small change in pH value can be translated to a greater change in the concentration of lactic acid in meat (Blixt and Borch, 2002). The essence of the effect of pH on the growth kinetics of *Pseudomonas* may be its sensitivity to undissociated levels of lactic acid in meat (Koutsoumanis et al., 2006). The growth of other spoilage bacteria including *B. thermosphacta* can also be affected by pH (Koutsoumanis et al., 2006). McClure et al. (1993) observed the growth of *B. thermosphacta* in broth medium at pH 5.6–6.8. In the study of Leroi et al. (2012), the optimal and minimal pH of *B. thermosphacta* was determined to be approximately 7.0 and 4.8, respectively. Conversely, there is usually no significant difference in growth of LAB under different pH values (Koutsoumanis et al., 2006; Koutsoumanis et al., 2004), which may be attributed to the higher acid tolerance of these

bacteria (Koutsoumanis et al., 2004). The spoilage activities of *Clostridium frigoris* and *C. estertheticum* seem to be pH-dependent; *C. frigoris* and *C. estertheticum* caused package swelling of beef samples at pH of 5.7–5.9 and 5.4–5.9, respectively, and neither led to blown-pack spoilage of high-pH ($\geqslant$ 6.0) beef samples (Yang et al., 2014a).

**Temperature.** Temperature is one of the main strategies in extending shelf-life of food products (Clemens et al., 2010). The lowest recommended storage temperature for meat products without freezing is -1.5$^{o}$C (Hernandez-Macedo et al., 2011). Relatively low temperature is able to extend lag phase duration, reduce growth rate, and even reduce final bacterial populations (Chaves et al., 2012; Doulgeraki et al., 2012; Li et al., 2013a; Mataragas et al., 2006). However, VP beef products are often stored at temperatures higher than -1.5$^{o}$C; up to as much as 10$^{o}$C (Koutsoumanis et al., 2006).

In the study of Youssef et al. (2014a), the sensory analysis of VP top butt cuts indicated acceptable sensory characteristics were found after storage for 140 and 160 days for samples stored at 2$^{o}$C and -1.5$^{o}$C, respectively. Temperature might also affect the microbial composition of the beef samples used in this study, in which *C. maltaromaticum* was the only LAB species recovered from cuts stored at -1.5$^{o}$C, while *C. maltaromaticum* and *C. divergens* were both recovered from cuts stored at 2$^{o}$C.

In meat, temperature not only influences the growth of microbiota but also affects their spoilage-related activity (Ercolini et al., 2009). The ability of microorganisms to adapt to temperature differs among species. *Pseudomonas* spp. are usually connected with the spoilage of meat at chill temperatures under aerobic atmospheres (Ercolini et al., 2010; Ercolini et al., 2007; Labadie, 1999), while for VP chilled meat, psychrotrophic LAB and *Clostridium* spp. are often involved in spoilage (Borch et al., 1996; Shaw and Harding, 1984).

*Enterobacteriaceae*, *Pseudomonas* spp. and *Acinetobacter* spp. are prevalent in the spoilage microbiota at an abuse temperature of 30°C (Gill and Newton, 1978).

**Other factors.** Other factors may affect VP beef spoilage, for example, beef nutrient composition, water activity, and bacterial interactions. Since the $a_w$ of meat is relatively high, studies of the influence of $a_w$ on growth of spoilage bacteria on meat are limited (Borch et al., 1996). Interactions among bacteria of VP beef also likely influence beef spoilage rates. In the study of Youssef et al. (2014a), only *C. maltaromaticum* and mostly consisting of only one strain of this species, was isolated from beef cuts stored at -1.5$^{o}$C, indicating the growth of other LAB species or strains may have been suppressed by this *C. maltaromaticum* isolate. The unusually long shelf-life reported by Youssef et al. (2014a) and Small et al. (2012) may be related to an unusual microbial community composition and interactions among the bacteria on their VP beef samples. To date, relevant information on the influence of microbial interactions on stored meat surfaces remains limited.

## BACTERIAL INTERACTIONS

**Bacterial interactions in nature and foods.** It has been recognized that bacteria normally act collectively rather than individually in nature (Faust and Raes, 2012; Keller and Surette, 2006). Beside environmental factors, inter- or intra- species interactions play an important role in shaping the ultimate bacterial community structure (Aguirre-von-Wobeser et al., 2014; Aziz et al., 2015; Giaouris et al., 2015; Perez-Gutierrez et al., 2013). Competition and cooperation are two main forms of interactions.

Interactions among a few food sourced bacterial species have been reported in the literature, and these interactions are considered to affect food safety or spoilage. The emphasis of these studies is mainly on the inhibitory activity of LAB on pathogenic or

spoilage bacteria *in vitro* or *in situ* within food (Metaxopoulos et al., 2002; Ostergaard et al., 2014; Vereecken et al., 2000; Vermeiren et al., 2006). For example, Ostergaard et al. (2014) contemplated the inhibiting effect of LAB when developing a growth model for *Listeria monocytogenes* in cottage cheese. In the study of Metaxopoulos et al. (2002), LAB exhibited inhibitory effects on the growth of spoilage microflora in cooked cured meat products. A few researchers have also observed the inhibitory effect of natural microbiota of meat on the growth of pathogens, which has been defined as the 'Jameson effect' (Cornu et al., 2011; Jameson, 1962; Moller et al., 2013; Ross et al., 2000; Vermeiren et al., 2006). The growth of *E. coli* was affected by the population density of competing microorganisms in ground beef (Coleman et al., 2003). Studies regarding the effect of natural microbiota on the growth of *Salmonella* have been performed in ground chicken and pork (Moller et al., 2013; Oscar, 2006, 2007; Zaher and Fujikawa, 2011). However, different from the Jameson effect, the presence of *Pseudomonas* spp. was found to enhance the survival of the pathogenic species *Campylobacter jejuni in vitro* and in poultry (Balamurugan et al., 2011; Hilbert et al., 2010).

Both growth inhibition and promotion interactions among spoilage related bacteria were observed in the study of Joffraud et al. (2006), in which cold-smoked salmon fillets were inoculated with pure or mixed cultures of *C. maltaromaticum*, *Photobacterium phosphoreum*, *L. sakei*, a *Vibrio* sp., *B. thermosphacta*, and a *S. liquefaciens*-like strain; the spoilage activity of bacteria in mixed cultures was found to significantly differ from pure cultures. The abilities of *E. coli* and *Pseudomonas mirabilis* to produce biogenic amines in broth were respectively promoted and reduced by *B. thermosphacta* (Nowak and Czyzowska, 2011). The viable counts of *B. thermosphacta* were reduced in the presence of LAB on agar media (Russo et al., 2006). In the study of Youssef et al. (2014a), mostly only a single strain of *C. maltaromaticum* was found on chilled VP beef cuts, potentially indicating the growth of other bacteria was suppressed by antibacterial compounds produced by this strain.

Compared to the number of bacterial interaction reports dealing with food safety issues, reports on the interactions of spoilage related microorganisms associated with meat, especially on VP beef, are even scarcer. Also, these interaction studies involve a limited number of bacterial species. The investigation on interactions on a greater range of species is much needed.

**Bacterial interaction mechanisms.** *Competition-type interactions.* Competition may be displayed as direct nutrient competition. This type of competition is also called scramble competition or exploitation competition. Limited resources may be rapidly used by competitive bacteria without direct interaction (Hibbing et al., 2010). Bacteria also compete with neighbouring microorganisms by antagonism, by secreting compounds either deliberately or incidentally as part of their standard metabolism. For food-sourced bacteria, these compounds may be bacteriocins, organic acids, hydrogen peroxide, and others (Faust and Raes, 2012; Oliveira et al., 2008; Vaughan et al., 2001).

Bacteriocins are ribosomally synthesized antibacterial heat-stable peptides that exhibit antagonistic activity against closely related bacteria, most of which are Gram-positive (Cotter et al., 2013). To date, most bacteriocin-producing isolates found in foods are LAB, hence, the potential of LAB as protective cultures has been extensively investigated in food products (Luchansky, 1999). Certain strains of *L. sakei* and *L. mesenteroides* have been reported to produce sakacins and mesenterocins, respectively (Vaughan et al., 2001). Also, a number of *C. divergens* and *C. maltaromaticum* isolates are able to produce bacteriocins that have wide inhibition spectra (Laursen et al., 2005). Interestingly, the bacteriocins produced by *C. divergens* and *C. maltaromaticum* often demonstrate intra-species inhibitory activity, for example, carnocyclin produced by *C. maltaromaticum* UAL 307 in the study of Martin-Visscher et al. (2008). These bacteriocins may have a strong impact on the community

structure of meat, especially when stored anaerobically; however, the relevant information related to this is still scant (Youssef et al., 2014a).

There are other inhibitory compounds that may also contribute to competition among bacteria. LAB are able to produce hydrogen peroxide and organic acids, primarily lactic acid and acetic acid, which can suppress the growth and metabolism of other bacteria in the food environment (Corsetti et al., 1998; Lindgren and Dobrogosz, 1990; Vasilopoulos et al., 2010). Russo et al. (2006) attributed the inhibitory activity of LAB on the growth of *B. thermosphacta* to low pH due to lactic acid accumulation.

Cell-to-cell contact is necessary for a few bacterial species when competing with neighbouring bacterial cells (Avendano-Perez and Pin, 2013; Hayes et al., 2014; Russell et al., 2011). Aoki et al. (2005) described a contact-dependent growth inhibition system among *E. coli*, in which a cell-surface protein is involved. In serial passage experiments, *E. coli* K-12 with a hyper mutability phenotype (due to deletion of *mutS*) could inhibit the growth of analogous wild-type strains by physical contact when cells stopped growing; this was designated as stationary contact-dependent inhibition (Lemonnier et al., 2008). Dubey and Ben-Yehuda (2011) observed different sized tubular extensions between neighbouring cells under electron microscopy that could be used to transfer intracellular molecules. These types of nanotubes have been found between cells belonging to the same and different species. They believe that these nanotubes could be the main mechanism mediating bacterial communication in nature (Dubey and Ben-Yehuda, 2011). *Pseudomonas aeruginosa* deliver bacteriolytic effectors to other Gram-negative bacterial cells via the type VI secretion delivery system (T6SS) (Russell et al., 2011). This secretion system has also been studied in *Vibrio cholerae* and *Burkholderia thailandensis*; furthermore, T6SS has been identified in > 80 Gram-negative bacterial genomes, including many members of the family *Enterobacteriaceae* (Boyer et al., 2009; MacIntyre et al., 2010; Russell et al., 2011; Schwarz

et al., 2010). According to Avendano-Perez and Pin (2013), live cells of human faecal bacteria were needed to inhibit *S. enterica* Typhimurium. Contact-dependent inhibition has been rarely reported in food research; however, a few studies suggest this type of bacterial interaction occurs in food systems. Schillinger and Lucke (1989), demonstrated that some strains of *L. sakei* isolated from meat inhibit the growth of some other lactobacilli on solid media, while their cell-free supernatants did not exhibit inhibitory activity. Similarly, 36 LAB isolates were shown by Oliveira et al. (2008) to inhibit the growth of American Type Culture Collection reference strains of lactobacilli, while the supernatants of only six strains exhibited inhibitory activity. This is probably a type of contact-dependent growth competition involving cell surface proteins, nanotubes, or other proteins and inter-cell structures. Further investigation is obviously needed to elucidate the exact mechanisms involved in the inhibitory activities of LAB.

*Cooperation.* Cooperation is another type of bacterial interaction, which is very common in nature. Bacteria of two species may exchange metabolic products to benefit the growth of each other (Faust and Raes, 2012; Pande et al., 2015; Ponomarova and Patil, 2015). Also, one bacterial species may promote the growth of others by increasing nutrient availability. For instance, siderophores, which help to improve access to iron in the environment, can be utilized as "public goods" within a bacterial community (Buckling et al., 2007). According to D'Onofrio et al. (2010), previously uncultured bacteria from marine sediment were able to grow on agar in the presence of a readily cultured bacteria due to the latter forming a growth promoting siderophore. *Pseudomonas* spp. have been reported to enhance the growth of other strains in chicken meat via producing biosurfactants and making nutrients more freely available (Mellor et al., 2011).

A large number of bacteria have been known to regulate their cooperative activities through quorum sensing systems (QS). They communicate by producing, detecting, and

responding to small diffusible signalling molecules which are defined as auto inducers (AI) (Giaouris et al., 2015; Skandamis and Nychas, 2012). These signalling molecules have been classified into four categories. AI-1 are N-acyl homoserine lactones, which are produced by Gram-negative bacteria and utilized for intra-species communication; while AI-2, furanosyl borate diesters are produced by Gram-negative and Gram-positive bacteria and have been regarded as a universal language for intra- and inter-species communication; AI-3, unknown aromatic compounds, are specific for pathogenic *E. coli* and used to detect epinephrine-producing host cells; AI-4 are auto inducing peptides (AIPs) produced by various Gram-positive bacteria (Skandamis and Nychas, 2012).

QS has been well described in *Aliivibrio (Vibrio) fischeri*, a Gram-negative species (Miller and Bassler, 2001). As shown in Fig. 1, when *N*-(3-oxohexanoyl)-homoserine lactone (OHHL; AI-1) reaches a threshold concentration outside of the cell, LuxR (transcription factor) interacts with OHHL and binds to the upstream of *luxICDABE,* and induces the transcription of proteins of the luciferase system (Gobbetti et al., 2007). The transcription of *luxI* gene encoding OHHL synthase is also up-regulated at the same time.

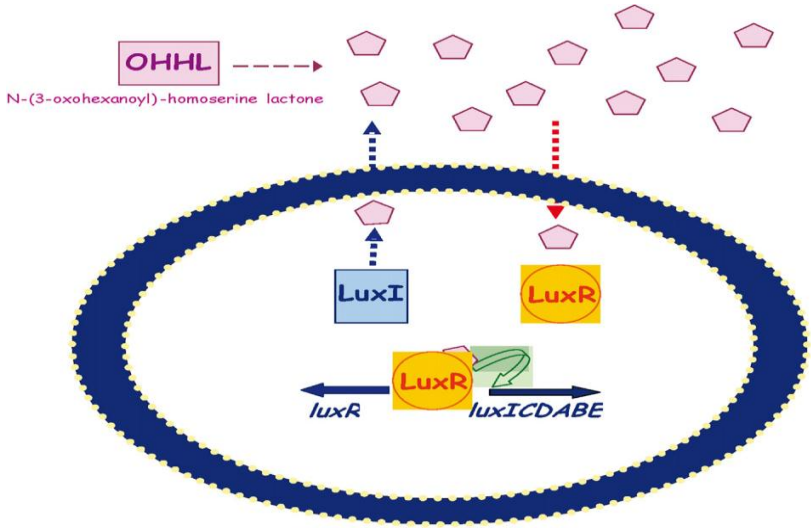



**FIG 1** Quorum sensing in *Aliivibrio* (*Vibrio) fisheri.* LuxI, auto inducer synthase; LuxR, transcription factor; *luxICDABE*, luciferase structural operon. Cited from March and Bentley (2004).

There are some food-related Gram-negative bacteria shown to possess the LuxI/LuxR-like QS system. For example, *Enterobacter agglomerans* uses EagI/EagR as its regulatory proteins, and *N*-(3-oxohexanoyl)-HSL as its auto inducer. *Enterobacteriaceae*, especially *H. alvei* and *Serratia* spp. have been frequently reported to produce AI-1, mainly OHHL (Blana and Nychas, 2014; Bruhn et al., 2004; Gram et al., 2002; Ravn et al., 2001; Silva et al., 2002). *Yersinia enterocolitica* has YenI/YenR as regulatory proteins, and *N*-hexanoyl-HSL and *N*-(3-oxohexanoyl)-HSL as auto inducers (Swift et al., 1993; Throup et al., 1995). *N*-butanoyl-HSL is the auto inducer of the QS system in *S. liquefaciens* (Eberl et al., 1996; Givskov et al., 1998). Onilude et al. (2002) hypothesized that *Pseudomonas* spp. can form biofilms in meat and that QS may be involved in this process.

The QS in Gram-positive bacteria usually involves a three-component regulatory system (3CRS) and is very different from the QS system of Gram-negative bacteria (Gobbetti et al.,

2007). This 3CRS gene cassette includes genes for AIP that is a ribosomal-generated oligopeptide, histidine protein kinase (HPK) and a response regulator (RR) (Hellingwerf et al., 1998; Samelis et al., 2003). As these two proteins are located on the outer surface of the cytoplasmic membrane, AIP do not need to internalize to produce a communication signal (Gobbetti et al., 2007). Nakayama et al. (2003) acquired putative amino acid sequences of HPK for some species of *Lactobacillus, Enterococcus* and *Clostridium* and these sequences show high similarity with other members of the $HPK_{10}$ subfamily. It has also been found that bacteriocin synthesis in *Lactobacillus plantarum, L. sakei* (Brurberg et al., 1997; Eijsink et al., 1996), *Enterococcus feacium* (O'Keeffe et al., 1999) and *C. maltaromaticum* (Quadri et al., 1997) is controlled by 3CRS.

It is interesting that both Gram-positive and Gram-negative quorum sensing mechanisms are present in *Vibrio harveyi* (Bassler et al., 1994). AI-1 and sensor 1 (LuxN) compose system 1 involved in intra-species quorum sensing; however system 2, made up of sensor 2 (LuxPQ) and AI-2, can be used for interspecies cell-cell communication (Surette et al., 1999). Database analysis has indicated that highly conserved homologues of *luxS,* the gene for synthesis of AI-2, exist in many Gram-negative and Gram-positive bacteria (Surette et al., 1999; Xavier and Bassler, 2003). AI-2, as a potential universal language used for both intra- and interspecies communication, possesses the ability to modulate the gene expression of diverse bacterial species and genera (De Keersmaecker et al., 2006; Lonn-Stensrud et al., 2007; Taga, 2005).

Cell-free supernatants (CFS) of foods are often extracted to determine AI-2 or ‘AI-2 like activity’ and to better understand QS activity. Bassler et al. (1993) first designed *V. harveyi* strain BB170 that can be applied to detect AI-2-like activity in food samples by its sensor Lux PQ. Sivakumar et al. (2011) also described a detailed protocol to detect AI-2-like activity using CFS. This activity is present in some foods like frozen fish, tofu and some vegetables,

while it is absent in others such as, uncooked frozen beef patties, uncooked beef steak and uncooked chicken breast (Lu et al., 2004, 2005). A possible explanation for these results is that AI-2 bioassay is inhibited by some compounds present in meat or poultry products (Soni et al., 2008; Widmer et al., 2007), while further study should still be done to explicate this phenomenon.

In the study of Ferrocino et al. (2009), all 72 strains of *P. fragi* isolated from fresh and spoiled meat produced AI-2 but could not produce N-acyl homoserine lactones. Hence, they believe that the QS system of *P. fragi* in meat is not regulated by N-acyl homoserine lactones, and AI-2 may play a role in meat spoilage dynamics. LAB and *B. thermosphacta*, as Gram-positive bacteria, are capable of producing AI-2 and/or AI-4; however, the production level was not sufficient to induce biosensor strains applied in the assay (Schaefer et al., 2000; Sturme et al., 2002). Nychas et al. (2009) first determined the effect of natural QS compounds (AI-1 and AI-2) on the kinetic parameters of *Serratia marcescens* and *Pseudomonas fluorescens in vitro*, both of which are main spoilage organisms in pork. They found that the growth rate of these two bacteria could be increased by these QS compounds.

The potential role of QS in food spoilage has been highlighted; however, the relevant literature is far from being conclusive. The mechanisms of action of QS compounds in interactions among spoilage bacteria, which specifically influence the spoilage process of foods, still need to be revealed. Also, the effect of natural compounds in foods on the activity of QS compounds is not clear.

## POTENTIAL FACTORS INFLUENCING INTERACTIONS ON VP BEEF

Various environmental factors of meat, including intrinsic factors, pH and meat nutrients (e.g. glucose), and extrinsic factors such as atmosphere and temperature, may affect interactions among bacterial communities on meats. A number of researchers have investigated the effect of these factors on the production or inhibitory activity of bacteriocins, which are important antagonistic compounds in competition interactions of foods, as mentioned above.

**Intrinsic factors**. Relatively low pH may induce *C. maltaromaticum* to lose its capability of producing bacteriocins; for example, *C. maltaromaticum* LV61 did not produce bacteriocin at pH 5.5 in the study of Schillinger et al. (1993); Khouiti and Simon (2004) observed that strain 213 could not produce carnocin in MRS medium at a pH below 6.5. pH also affects the inhibitory activity of bacteriocins (Abriouel et al., 2001). Nisin, sakacin P, and curvacin A demonstrate relatively greater inhibitory activity at low pH in broth media (Balciunas et al., 2013; Ganzle et al., 1999). As an important carbon source, the concentration of glucose in foods can also affect the production of bacteriocins (Biswas et al., 1991; Khouiti and Simon, 2004; Vignolo et al., 1995). Moreover, the target bacterial cells are able to be energized by the presence of glucose and their sensitivity to bacteriocins then may be enhanced (Castellano et al., 2003; McAuliffe et al., 1998).

**Extrinsic factors.** The effect of other factors on bacteriocin production or activity has received far less attention. Regarding meat packaging atmosphere, it has been reported that the production of lactocin by *L. sakei* L45 was best under anaerobic conditions (McAuliffe et al., 1998). As small peptides, bacteriocins may be sensitive to proteases existing in meats. Aasen et al. (2003) found that proteolytic activity induced bacteriocin degradation in raw chicken. A specific temperature range has been reported to be required by certain *C.*

*maltaromaticum* isolates to produce bacteriocins, for instance, bacteriocin activity was only observable in the supernatant of *C. maltaromaticum* UAL26 grown in liquid media at temperatures less than 19°C (Gursky et al., 2006).

Other types of interactions may also be affected by aforementioned meat factors; however, the relevant information is quite limited and piecemeal. According to Speranza et al. (2010), packaging atmosphere affects interaction between *P. putida* and *E. coli* O157:H7, with interaction being stronger in modified atmosphere packaging compared to aerobic conditions. QS signal molecules were found to be affected by the packaging conditions e.g. temperature and atmosphere used for meat preservation as a consequence of the development of a distinct microbial community (Blana and Nychas, 2014).

## PREDICTIVE MODELS CONSIDERING BACTERIAL INTERACTIONS

Predictive microbiology is a discipline to study microorganism behaviour as a function of different intrinsic or extrinsic environmental parameters of foods (Cavre et al., 2005; McMeekin et al., 1987). It is a proven beneficial tool to evaluate food safety and shelf-life (Castillejo-Rodriguez et al., 2002; Dominguez and Schaffner, 2007; Mataragas et al., 2006).

In the past 20 years, extensive predictive models on the growth of foodborne pathogens and spoilage bacteria of meat have been developed (Dalgaard, 1995; Koutsoumanis et al., 2000; Mellefont et al., 2003; Oscar, 2002). Most of these models predict the growth of a single bacterial species or changes in the total bacterial population (Dalgaard, 1995; Koutsoumanis et al., 2000; Mellefont et al., 2003; Oscar, 2002). A few predictive growth models also considered bacterial interactions, primarily the ‘Jameson effect’ (Cornu et al., 2011; Gimenez and Dalgaard, 2004; Moller et al., 2013).

However, the ‘Jameson effect’ is only one of the situations involving a competition type interactions within a bacterial community, and in reality in the case of the meat environment, a large variety of bacteria and complicated interactions exist together. Due to lack of information on these interactions, they are seldom considered in developing predictive models. Relevant research will help to understand the nature of dynamic features of meat bacterial communities that may be composed of inhibiting, sensitive, or promoting isolates, which in turn can dictate the rate that meat spoilage occurs.

## CONCLUSIONS

VP beef is usually contaminated by diverse bacterial species during production, and contains different proportions of LAB, *Enterobacteriaceae, B. thermosphacta*, *Pseudomonas*, and *Clostridium* due to the subsequent selection pressure of the packaging. The long shelf-life (26–30 weeks) of Australian VP beef may be partly attributed to a specific composition of bacteria and the interactions amongst them, in addition to good hygienic processing practices.

Bacterial interactions may be the underlying forces that shape community structural changes in food, which then affect shelf-life. However, the studies on interactions among meat-sourced bacteria have so far received limited attention. Further, these limited interaction studies have included relatively small number of strains/species, warranting broadening the range of taxa that need to be included, due to the complexity of bacterial communities on meats.

Moreover, bacteria cooperate or compete in a community by various mechanisms. A number of researchers have studied protective cultures producing inhibitory compounds, e.g.

LAB. Other interaction mechanisms are not well explored in food, for example, whether contact-dependent inhibition exists in meat bacterial community.

Furthermore, how environmental factors affect bacterial interactions has not received any research effort. Interactions are seldom considered when developing bacterial growth models due to limited understanding. However, relevant research may help develop improved strategies to maintain/extend shelf-life of meats.

# Chapter 3

# Interstrain interactions between bacteria isolated from vacuum-packaged refrigerated beef


## ABSTRACT

The formation of bacterial spoilage communities in food is influenced by both extrinsic and intrinsic environmental factors. While many reports describe how these factors affect bacterial growth, much less is known about interactions among bacteria, which may influence community structure. This study investigated interactions among representative species of bacteria isolated from vacuum-packaged (VP) beef. Thirty-nine effectors and 20 target isolates were selected, representing 10 bacterial genera: *Carnobacterium*, *Pseudomonas*, *Hafnia*, *Serratia*, *Yersinia*, *Rahnella*, *Brochothrix*, *Bacillus*, *Leuconostoc* and *Staphylococcus*. The influence of live effectors on growth of target isolates was measured by spot-lawn agar assay, and also in liquid culture medium broth using live targets and effector cell-free supernatants. Inhibition on agar was quantified by diameter of inhibition zone, and in broth by measuring detection time, growth rate, and maximum population density. A number of interactions were observed, with 28.6% of isolates inhibiting and 4.2% promoting growth. The majority of *Pseudomonas* isolates antagonised growth of approximately one-half of target isolates. Two *Bacillus* spp. each inhibited 16 targets. Among lactic acid bacteria (LAB), *Carnobacterium maltaromaticum* inhibited a wider range of isolates compared to other LAB. The majority of effector isolates enhancing target isolate growth were Gram-negative, including *Pseudomonas* spp. and *Enterobacteriaceae*. These findings markedly improve the

understanding of potential interactions among spoilage bacteria, possibly leading to more mechanistic descriptions of bacterial community formation in VP beef and other foods.

## INTRODUCTION

The shelf-life of meat is influenced, in part, by the composition and levels of bacteria within the spoilage community (Nychas et al., 2008). Independent laboratories have confirmed relatively high microbial diversity at the time of meat packaging, and showing a progressive shift to lower community complexity towards the end of shelf-life (De Filippis et al., 2013; Powell and Tamplin, 2012; Sakala et al., 2002). For refrigerated vacuum-packaged (VP) beef, over time and under best-practice conditions, lactic acid bacteria (LAB) tend to predominate and, to a lesser extent, *Enterobacteriaceae* (Doulgeraki et al., 2012).

Such change in bacterial community structure is based on intrinsic and extrinsic factors, including temperature, atmosphere, pH, and organic acids, all of which may influence growth (Doulgeraki et al., 2012; Nemergut et al., 2013). However, the underlying forces of microbial interactions may also be important in shaping biodiversity of communities (Blana and Nychas, 2014; Faust and Raes, 2012; Perez-Gutierrez et al., 2013; Wolfe et al., 2014); such studies have received relatively little attention in foods. Bacteria interact in any given ecological niche through different mechanisms including quorum sensing, contact-dependent inhibition, nutrient competition, and via production of defence compounds such as bacteriocins, antibiotics and organic acids (Aoki et al., 2005; Blana and Nychas, 2014; Cotter et al., 2013; Deriu et al., 2013; Dubey and Ben-Yehuda, 2011). There have been numerous reports exploring the effectiveness of protective cultures and related antibacterial compounds at enhancing food safety and extending shelf-life (Budde et al., 2003; Hastings et al., 1994; Hequet et al., 2007; Li et al., 2011), however, few have investigated interactions among food bacteria, and of those which have, relatively few species have been studied (Dourou et al.,

2011; Mellefont et al., 2008; Russo et al., 2006; Vasilopoulos et al., 2010); far fewer have involved species from diverse communities (Mounier et al., 2008; Wolfe et al., 2014).

Nychas et al. (2009) found quorum-sensing compounds extracted from meat increased the growth rate of *Serratia marcescens* and *Pseudomonas fluorescens*. Also, Russo et al. (2006) reported the growth of *Brochothrix thermosphacta*, a meat spoilage bacteria, decreased in the presence of LAB. We postulate testing a wide range of bacterial genera and species can provide a fuller understanding of potential complex interactions.

The spot-lawn agar method (Benkerroum et al., 1993) has been widely used to detect bacterial inhibitory activity, via reporting an inhibition zone (Aguirre-von-Wobeser et al., 2014; Lo Giudice et al., 2007; Perez-Gutierrez et al., 2013). However, this method does not supply specific information about the effect of an effector on target growth, such as that achieved using broth-based assays. Also, the latter assay more readily detects growth-promotion among isolates (Nychas et al., 2009).

In this study, we applied both spot-lawn agar assay and broth assay, and investigated interactions among a diverse group of bacteria isolated from VP beef produced at six Australian abattoirs. Network maps illustrate the complexity of interactions, and the possible role of specific bacterial genera in community structure. Such information might eventually be translated into models describing dynamic changes in bacterial communities, and better inform processing and preservation strategies to enhance meat quality and shelf-life.

## MATERIALS AND METHODS

**Bacterial isolates.** The 180 bacterial isolates used in this study were previously obtained from VP beef primals produced at six Australian abattoirs, stored at -0.5°C, and sampled at

various time intervals for up to 30 weeks, as described by Small et al. (2012). Ten colonies, representing different morphologies, were obtained and stored at -80°C. The isolates were identified by 16S rRNA gene sequences amplified using universal primers 10F (5'-GAGTTTGATCCTGGCTCAG -3') and 907R (5'-CCGTCAATTCCTTTGAGTTT-3'). The PCR products were sent to Macrogen (Seoul, Korea) for sequencing. Sequences were compared with those in Genbank using the BLAST function (http://blast.ncbi.nlm.nih.gov/Blast.cgi), and the closest matches of each clone determined specific probable identities.

The 180 isolates were screened for inhibitory activity by using a spot-lawn method (Benkerroum et al., 1993) at 25°C under aerobic conditions. Thirty-nine of the isolates showing inhibition (effectors) were selected, representing different species, abattoirs, storage times, and bacterial genera (Table 1). Twenty target (inhibited) bacteria were selected using the same criterion as effector bacteria (i.e., different species, abattoirs, storage times, and bacterial genera). Effector and target isolates comprised 10 genera, i.e., *Carnobacterium*, *Pseudomonas*, *Brochothrix*, *Hafnia*, *Yersinia*, *Bacillus*, *Rahnella*, *Leuconostoc*, *Serratia and Staphylococcus* (Table 1 and 2). Six (*Leuconostoc mesenteroides* B30b, *Staphylococcus epidermidis* F30c, *Bacillus* sp. strain A30g, *Pseudomonas* sp. D0g, *Yersinia enterocolitica* B8b and *Rahnella aquatilis* B8f) were tested as both targets and effectors. The rationale for isolate selection was not based on the species observed in a specific package of VP beef (24) but instead on having a panel of isolates representing those species found in VP beef from different abattoirs. Isolates were stored at -80$^{o}$C in brain heart infusion broth (BHI; Amyl Media Ltd., Australia), supplemented with 20% (vol/vol) glycerol.

**TABLE 1** Growth inhibition and promotion activity for effector isolates, as tested by spot-lawn and CFS assays

| Effector | Isolate code | Inhibition (no.)[a] | | | | | Promotion (no.)[a] |
|---|---|---|---|---|---|---|---|
| | | Agar[b] | | CFS[c] | | | |
| | | Targets | Inhibited | Targets | Inhibited | Total[d] | |
| *Carnobacterium divergens* | A0a | 20 | 2 | 19 | 4 | 5 | 0 |
| | A0f | 20 | 2 | 19 | 4 | 5 | 0 |
| | C8j | 20 | 2 | 19 | 7 | 8 | 0 |
| | D30a | 20 | 1 | 19 | 3 | 3 | 0 |
| | E0j | 20 | 2 | 19 | 5 | 5 | 0 |
| | F8f | 20 | 2 | 19 | 2 | 4 | 0 |
| *Carnobacterium maltaromaticum* | B0f | 20 | 3 | 19 | 7 | 8 | 0 |
| | C0a | 20 | 0 | 19 | 5 | 5 | 0 |
| | C8h | 20 | 0 | 19 | 9 | 9 | 0 |
| | C30h | 20 | 3 | 19 | 9 | 9 | 0 |
| | D0h | 20 | 4 | 19 | 9 | 10 | 0 |
| *Carnobacterium* sp. | F8g | 20 | 0 | 19 | 7 | 7 | 0 |
| *Leuconostoc carnosum* | F30d | 20 | 0 | 19 | 2 | 2 | 0 |
| | F30h | 20 | 0 | 19 | 2 | 2 | 0 |
| *Leuconostoc mesenteroides* | B30b | 19 | 0 | 18 | 3 | 3 | 1 |
| *Brochothrix thermosphacta* | A8f | 20 | 0 | 19 | 5 | 5 | 0 |
| *Staphylococcus epidermidis* | F30c | 19 | 7 | 18 | 4 | 7 | 0 |
| *Bacillus subtilis* | E0g | 20 | 12 | 19 | 12 | 16 | 1 |
| *Bacillus* sp. | A30g | 19 | 14 | 18 | 6 | 16 | 2 |
| *Pseudomonas fluorescens* | B0i | 20 | 3 | 19 | 4 | 6 | 2 |
| | C0c | 20 | 8 | 19 | 3 | 9 | 1 |
| *Pseudomonas fragi* | F0b | 20 | 12 | 19 | 2 | 13 | 2 |
| *Pseudomonas putida* | D0b | 20 | 18 | 19 | 1 | 18 | 2 |
| *Pseudomonas* sp. | D0g | 19 | 10 | 18 | 1 | 10 | 1 |
| | E0f | 20 | 11 | 19 | 2 | 12 | 2 |
| *Hafnia alvei* | A8e | 20 | 1 | 19 | 0 | 1 | 0 |
| | D0f | 20 | 1 | 19 | 0 | 1 | 1 |
| | E30e | 20 | 0 | 19 | 0 | 0 | 1 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| *Yersinia enterocolitica* | B8b | 19 | 1 | 18 | 3 | 4 | 0 |
| *Yersinia frederiksenii* | A8h | 20 | 3 | 19 | 0 | 3 | 1 |
| *Yersinia* sp. | A8d | 20 | 3 | 19 | 0 | 3 | 0 |
| *Rahnella aquatilis* | B8f | 19 | 0 | 18 | 1 | 1 | 1 |
| *Serratia* sp. | C0b | 20 | 1 | 19 | 0 | 1 | 3 |
| | C30b | 20 | 3 | 19 | 0 | 3 | 2 |
| | E8i | 20 | 2 | 19 | 0 | 2 | 1 |
| | E8c | 20 | 3 | 19 | 0 | 3 | 4 |
| | E30g | 20 | 1 | 19 | 0 | 1 | 2 |
| | E30h | 20 | 0 | 19 | 0 | 0 | 0 |
| | E30j | 20 | 1 | 19 | 0 | 1 | 1 |

[a] no, the number of target isolates that were inhibited or promoted by each effector isolate.

[b] Agar, spot-lawn assay with live cells on agar.

[c] CFS, CFS-broth assay with cell-free supernatant in BHI broth.

[d] That is, the total number of unique inhibitions observed by spot-lawn and CFS assays.

**TABLE 2** Effectors inhibiting or promoting growth of target isolates

| | | %[a] | |
|---|---|---|---|
| Target | Isolate code | Inhibition | Promotion |
| *Carnobacterium divergens* | D30f | 51.3 | 25.6 |
| *Carnobacterium maltaromaticum* | D8c | 48.7 | 25.6 |
| *Hafnia alvei* | E30d | 17.9 | 0 |
| *Brochothrix thermosphacta* | A0b | 43.6 | 5.1 |
| *Yersinia enterocolitica* | B8b | 21.1 | 0 |
| *Yersinia* sp. | D8b | 25.6 | 0 |
| *Bacillus subtilis* | B30a | 25.6 | 0 |
| *Bacillus* sp. | A30g | 36.8 | 5.3 |
| *Serratia* sp. | B0h | 5.1 | 0 |
| *Serratia* sp. | D0c | 17.9 | 0 |
| *Serratia* sp. | D0d | 23.1 | 0 |
| *Pseudomonas lundensis* | D8g | 23.1 | 12.8 |
| *Pseudomonas fluorescens* | D8d | 33.3 | 0 |
| *Pseudomonas* sp. | D0g | 47.4 | 5.3 |
| *Staphylococcus saprophyticus* | E0c | 38.5 | 0 |
| *Staphylococcus epidermidis* | F30c | 44.7 | 0 |
| *Rahnella aquatilis* | B8f | 13.2 | 0 |
| *Leuconostoc carnosum* | F30j | 30.8 | 0 |

| | | | |
|---|---|---|---|
| *Leuconostoc mesenteroides* | B30b | 15.8 | 0 |
| *Leuconostoc* sp. | F30e | 7.7 | 0 |

[a] The percentages of target isolates where growth was inhibited or promoted are indicated.

**Inhibition activity measured on agar.** The spot-lawn method described by Benkerroum et al. (1993) was used to test for inhibitory activity of live effectors on target isolates. Briefly, all isolates were transferred from -80°C, streaked on tryptone soya agar (TSA; Oxoid Ltd., Australia), cultured for 24 h at 25°C, and then grown in BHI broth for 24 h at 25°C. Cell density was adjusted to an optical density at 540 nm ($OD_{540}$) 0.6-0.8 for effectors and 0.15-0.25 for targets, a difference designed to enhance detection of growth inhibition or promotion. One hundred microliters of each target was spread-plated on TSA, and then three replicate 10-µl aliquots of effectors were spotted onto the target lawn. Inhibition was measured after 24 h of incubation at 25$^{o}$C, when TSA plates were photographed, and the diameter (D) of the inhibition zone was measured using the software program Image J (version 1.49 [http://rsb.info.nih.gov/ij/index.html]). The degree of inhibition was classified at four levels: ++++, +++, ++, and +, corresponding to $D \geq 4$ mm, $2 \leq D < 4$ mm, $0.5 < D < 2$ mm and $0 < D \leq 0.5$ mm, respectively (Fig. 1). This grouping considered variation in inhibition strength and facilitated comparison. Inhibition patterns were also classified as having a well delineated or diffuse edge.

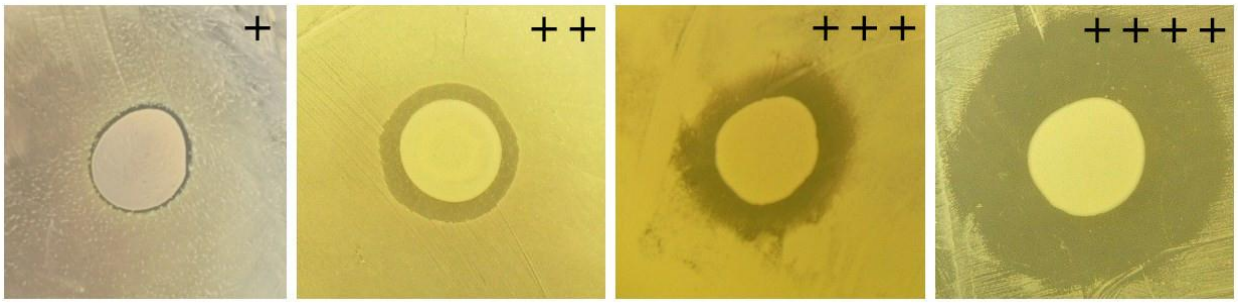


**FIG 1** Representative growth inhibition as determined by spot-lawn assay.

Inhibition of target isolates was determined to be at four levels, ++++, +++, ++, and +, corresponding to $D \geq 4$ mm, 2 mm $\leq D < 4$ mm, 0.5 mm $< D < 2$ mm and $0 < D \leq 0.5$ mm, respectively.

**Interaction activity measured by CFS assay.** Overnight cultures (24 h, 25$^{o}$C) of target isolates were adjusted to $10^4$ cfu/ml. Effector isolates were incubated for 48 h at 25$^{o}$C until the stationary phase was reached. Cell-free supernatant (CFS) of each effector isolates were made by centrifuging BHI cultures at 1,000 x *g* for 5 min, followed by filtration through a 0.22 µm pore-sized filter (Whatman Ltd., Australia). Treatments consisted of mixing 100 µl of the diluted target suspension with 100 µl of CFS in wells of a BioscreenC microwell plate (Growth Curve Ab Ltd., Finland). Controls had the same volume of fresh BHI or phosphate-buffered saline (PBS; 1M, [pH 7.4]), instead of CFS. Duplicate wells were used for all treatments and controls. The BioscreenC temperature was 25$^{o}$C, and growth kinetics measured at 20-min intervals for 48 h. At the end of each run, data were exported to an Excel spreadsheet. Detection time (DT; in hours) was calculated as the time to reach an $OD_{540}$ of 0.12 (background corrected data). The Baranyi model (Baranyi and Roberts, 1994) was fitted to the primary growth curves using DMFit (v3.0 [Combase; http://www.combase.cc/tools/]) to calculate growth rate (GR; $\log_{10}$OD/h). Maximum population density (MPD; $OD_{540}$) was calculated by averaging the three highest OD readings. DT, GR and MPD were compared among treatments and controls, using the Student t-Test in Excel. A *P* value below 0.05 was considered a significant interaction, i.e., as inhibition comparing treatment and PBS or as promotion comparing treatment and BHI.

If $P > 0.05$, inhibition strength (IS) of CFS on individual target growth parameter was recorded as zero. If $P < 0.05$, the IS was calculated by comparison of treatment and PBS control using the following formulas:

$$IS_{DT} = |DT_{Treatment} - DT_{Control}| / DT_{Control} \quad (1)$$

$$IS_{GR} = |GR_{Treatment} - GR_{Control}| / GR_{Control} \quad (2)$$

$$IS_{MPD} = |MPD_{Treatment} - MPD_{Control}| / MPD_{Control} \quad (3)$$

The cumulative IS effect ($IS_{Total}$) on all three growth parameters was quantified using the formula:

$$IS_{Total} = (IS_{DT} + IS_{GR} + IS_{MPD}) / 3 \quad (4)$$

The promotion strength (PS) was calculated similar to IS, via comparison of test and BHI control.

IS was classified at four levels, ++++, +++, ++, and +, corresponding to IS = 1 (no detectable growth of the target), $0.25 \leq IS < 1$, $0.15 \leq IS < 0.25$, and $0 < IS < 0.15$, respectively (Fig. 2). In the relatively fewer instances where CFS promoted growth, growth PS was classified at two levels, ++ and +, corresponding to $PS \geq 0.1$ and $0 < PS < 0.1$, respectively.

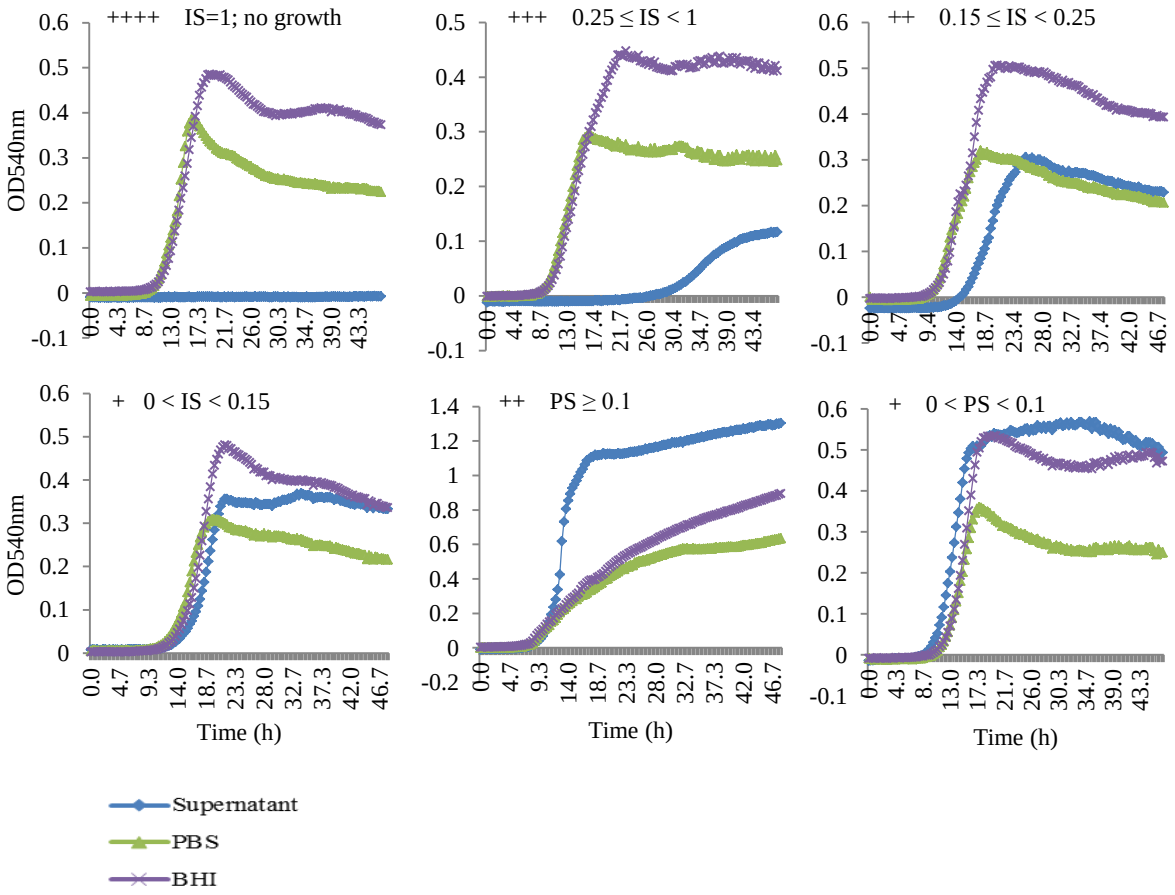



**FIG 2** Representative growth inhibition and promotion by CFS-broth assay.

**Network maps of bacterial interactions.** Growth inhibition/promotion activity was described using a network diagram drawn with Cytoscape (v3.1.1 [http://www.cytoscape.org/]) (Fig. 3). In maps, target and effector nodes were designated as diamonds and circles, respectively. Isolates used as both inhibitors and targets were represented by squares. Arrows (edges) connected interacting isolates. The strength of growth inhibition or promotion was distinguished by line thickness.

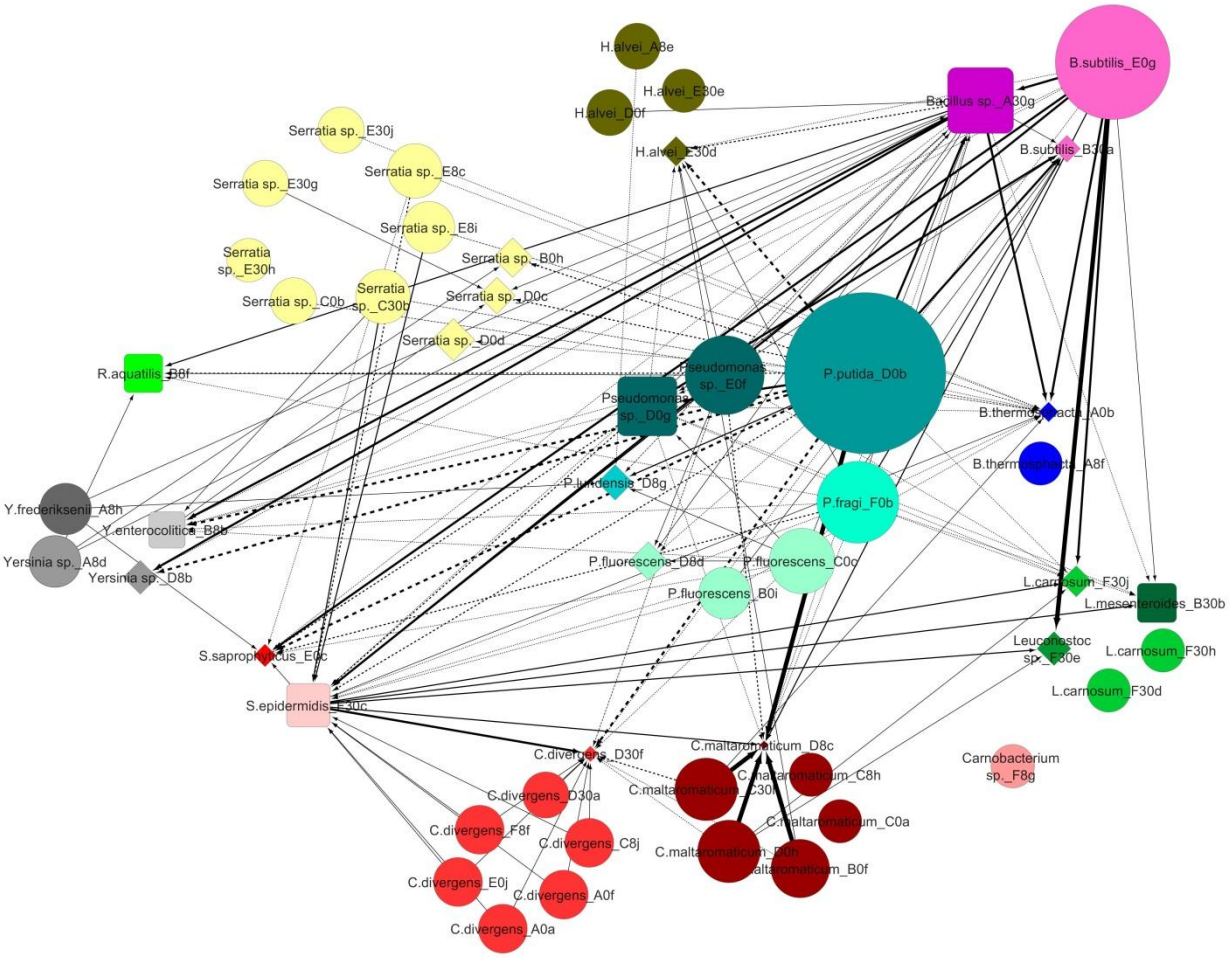
In terms of node size, an arbitrary base number (BN) of 80 was first assigned. Then, a connection number (CN) was calculated for each node according to the number of each interaction level as follows:

$$CN = \sum_{i=1}^{4} \left( \frac{a_i}{b} \times 100 \times i \right) \quad (5)$$

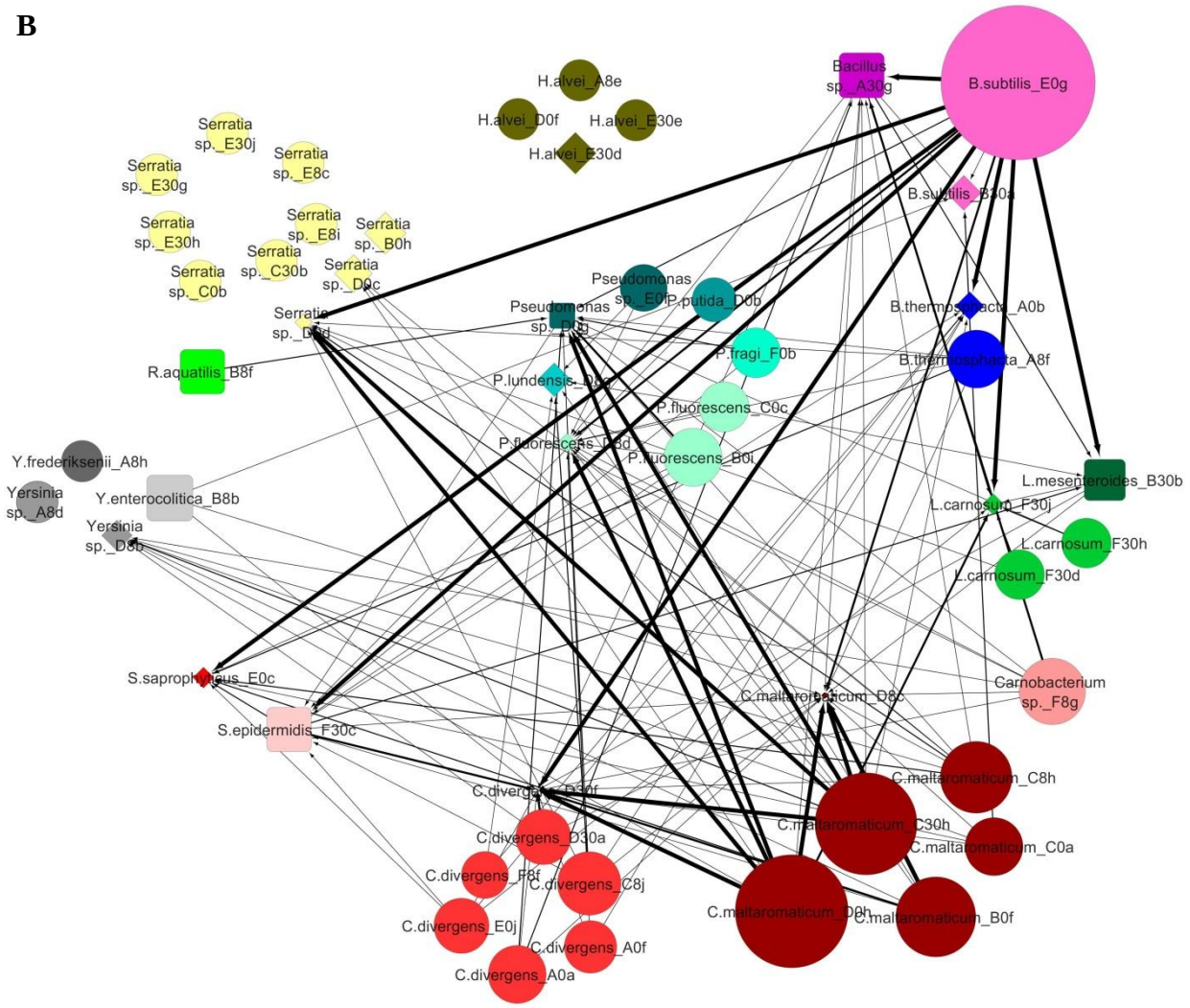
with *i* being the interaction level (1, +; 2, ++; 3, +++; and 4, ++++), $a_i$ the number of interactions at level *i*, and *b* the number of effectors or targets for corresponding target or effector.

In the growth inhibition network map, the size of individual inhibiting nodes equalled the sum of BN and CN. For target isolates, the diameter of the node was the difference between BN and CN; the smaller the diamond, the greater the target was inhibited. In growth promotion network maps, the size of both targets and effectors was set as the sum of BN and CN. For isolates being both a target and effector, node size was calculated as target and effector, respectively, and then the final size displayed as the average of these two values.

**A**


H.alvei_A8e
H.alvei_E30e
H.alvei_D0f
H.alvei_E30d
B.subtilis_E0g
Bacillus sp._A30g
B.subtilis_B30a
Serratia sp._E30j
Serratia sp._E8c
Serratia sp._E30g
Serratia sp._E8i
Serratia sp._B0h
Serratia sp._E30h
Serratia sp._C0b
Serratia sp._C30b
Serratia sp._D0c
Serratia sp._D0d
R.aquatilis_B8f
Pseudomonas sp._E0f
P.putida_D0b
Pseudomonas sp._D0g
B.thermoamylovorans_A0b
B.thermoamylovorans_A8f
P.lundensis_D8g
P.fragi_F0b
Y.frederiksenii_A8h
Y.enterocolitica_B8b
Yersinia sp._A8d
Yersinia sp._D8b
P.fluorescens_D8d
P.fluorescens_C0c
P.fluorescens_B0i
L.carnosum_F30j
L.mesenteroides_B30b
Leuconostoc sp._F30e
L.carnosum_F30h
L.carnosum_F30d
S.saprophyticus_E0c
S.epidermidis_F30c
C.divergens_D30f
C.maltaromaticum_D8c
C.maltaromaticum_C8h
C.maltaromaticum_C30f
C.maltaromaticum_C0a
C.maltaromaticum_B0f
Carnobacterium sp._F8g
C.divergens_D30a
C.divergens_F8f
C.divergens_C8j
C.divergens_E0j
C.divergens_A0f
C.divergens_A0a

**B**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H.alvei_D0f
H.alvei_E30e
H.alvei_E30d
Bacillus sp._A30g
B.subtilis_E0g
B.subtilis_B30a
Serratia sp._E30j
Serratia sp._E8c
Serratia sp._E30g
Serratia sp._E30h
Serratia sp._E8i
Serratia sp._B0h
Serratia sp._C30b
Serratia sp._C0b
Serratia sp._D0c
Serratia sp._D8d
Pseudomonas sp._E0f
P.putida_D0b
Pseudomonas sp._D8g
B.thermoamylovorans_A0b
B.thermosphaericus_A8f
P.fragi_F0b
R.aquatilis_B8f
P.lundensis_A0c
P.fluorescens_C0c
P.fluorescens_D8d
P.fluorescens_B0i
Y.frederiksenii_A8h
Yersinia sp._A8d
Y.enterocolitica_B8b
Yersinia sp._D8b
L.mesenteroides_B30b
L.carnosum_F30j
L.carnosum_F30h
L.carnosum_F30d
S.saprophyticus_E0c
S.epidermidis_F30c
C.maltaromaticum_D8c
Carnobacterium sp._F8g
C.maltaromaticum_C8h
C.divergens_D30f
C.maltaromaticum_C30h
C.maltaromaticum_C0a
C.divergens_D30a
C.divergens_F8f
C.divergens_C8j
C.maltaromaticum_D0h
C.maltaromaticum_B0f
C.divergens_E0j
C.divergens_A0f
C.divergens_A0a

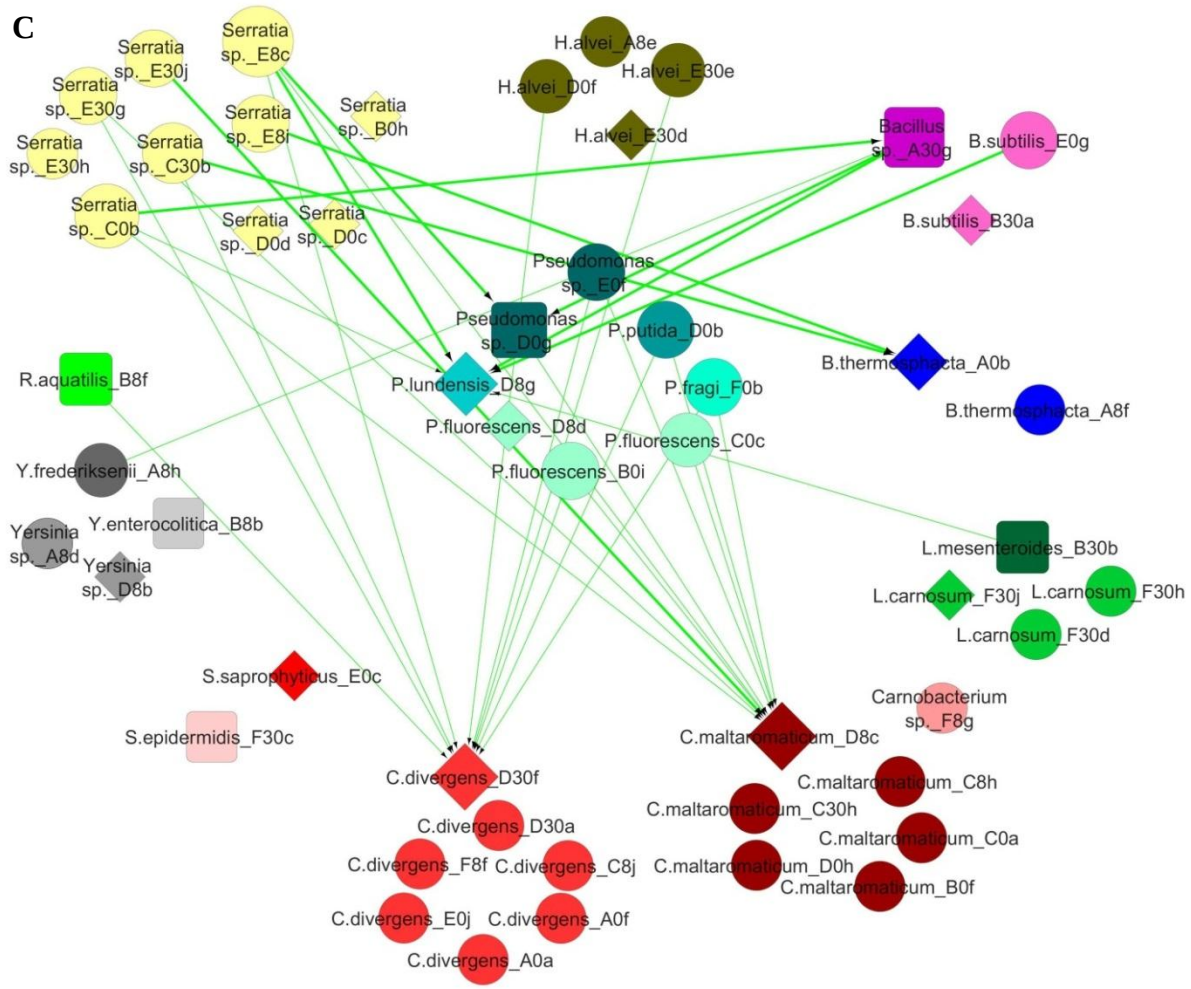



**FIG 3** Interactions among effector and target isolates.

(A) Inhibition, spot-lawn assay; (B) Inhibition, CFS assay; (C), Promotion, CFS assay. Symnols: ◇, target; ○, effector; ▢, isolate tested as both target and effector. a → b = a inhibited (A and B) or promoted (C) b. Thick to thin black (solid and dashed) arrows indicate "++++", "+++", "++", and "+" inhibition, respectively. Medium and thin green arrows indicate "++" and "+" growth promotion, respectively. Dashed and solid black arrows indicate diffuse and clear inhibition zones, respectively, in panel A. In panels A and B, the size of an effector and target node is, respectively, positively and negatively correlated with the number and level of inhibitions. In panel C, the size of both an effector and target node is positively correlated with the number and level of promotions.

**Statistical analysis.** The differences of distribution of growth-inhibiting and –promoting activity (IS and PS) among effectors at isolate, species and genus levels were statistically

analysed. An F-test was applied to examine overall differences among different groups. If the F-test was significant ($P < 0.05$), a Student *t* test was used to identify the significant pairwise differences. Differences between Gram-negative and -positive bacteria were also examined in the same way. The dependent variable in analysis included IS from spot-lawn assay (inhibition diameter, mm), and IS, PS, $IS_{DT}$, $IS_{GR}$, $IS_{MPD}$, $PS_{DT}$, $PS_{GR}$, and $PS_{MPD}$ from CFS assay (%). The arcsine transformation of square root of relative interaction strength was used to normalise the data from CFS assay. A *P* value below 0.05 from Student *t* test was considered statistically significant. These tests were performed using the GLM procedure in SAS (v 9.3; SAS, Inc., Rockville, MD).

## RESULTS

Total of 774 and 735 combinations of effector and target isolates were tested by spot-lawn and CFS assay, respectively. The difference of 39 (i.e. 774-735=39) in total combinations between the two assays resulted from *Leuconostoc* sp. F30e not sufficiently growing in BHI broth for CFS analysis.

**Summary of interactions**. Combined results of spot-lawn and CFS assays showed 31% of pairings produced an interaction, i.e., 28.6% (221 pairings) inhibitions compared to 4.2% (31 pairings) promotions. A slightly larger number of inhibitory reactions were detected by spot-lawn compared to CFS assay, i.e., 17.6% (136 pairings) versus 16.6% (122 pairings), respectively (Table 3).

**TABLE 3** Summary of growth inhibition and promotion activity

| Interaction level[a] | Spot-lawn assay (total) | | CFS assay[b] | | | |
|---|---|---|---|---|---|---|
| | Total | | Inhibition | | Promotion[c] | |
| | No. | % | No. | % | No. | % |
| ++++ | 6 | 0.8 | 19 | 2.6 | | |
| +++ | 17 | 2.2 | 7 | 1.0 | | |
| ++ | 21 | 2.7 | 17 | 2.3 | 9 | 1.2 |
| + | 92 | 11.9 | 79 | 10.7 | 22 | 3.0 |
| Total[d] | 136 | 17.6 | 122 | 16.6 | 31 | 4.2 |

[a] Spot-lawn assay: ++++, D ≥ 4 mm; +++, 2 mm ≤ D < 4 mm; ++, 0.5 mm < D < 2 mm; +, 0 < D ≤ 0.5 mm. CFS broth assay and growth inhibition: ++++, no growth of the target (IS =1); +++, 0.25 ≤ IS < 1; ++, 0.15 ≤ IS < 0.25; +, 0 < IS < 0.15. CFS assay and growth promotion: ++, PS ≥ 0.1; +, 0 < PS < 0.1.

[b] Effector cell-free supernatant.

[c] Growth promotion was classified at only two levels.

[d] That is the total number or percentage of effector-target pairings displaying inhibition or promotion among 774 and 735 effector-target parings studied by using spot-lawn and CFS assays, respectively.

**Growth inhibition**. Among the 774 effector-target pairings tested by spot-lawn assay, there were more weak (14.6%, + and ++) than strong inhibitions (3%, +++ and ++++) (Fig. 3 and Table 3). By CFS assay, 3.6% versus 13% of interactions produced strong versus weak inhibition, respectively. Analysis of kinetic growth profiles of target bacteria showed CFS primarily affected DT (Table 4), an effect particularly evident for *Carnobacterium* (Table A1: Appendix A). On the whole, more inhibition events were associated with increased DT (78.9% of inhibitions) than decreased GR (44.7%) and MPD (28.5%).

**TABLE 4** Effects on growth parameters measured by CFS assay

| | Inhibition | | Promotion | |
|---|---|---|---|---|
| Parameter[a] | %[b] | No. | % | No. |
| DT | 78.9 | 97 | 51.6 | 16 |
| GR | 44.7 | 55 | 32.3 | 10 |
| MPD | 28.5 | 35 | 29 | 9 |

[a] DT, detection time; GR, growth rate; MPD, maximum population density.

[b] The percentage was based on the number of interactions affecting a specific growth parameter, divided the total number of interactions (inhibition, 122; promotion, 31).

**Growth promotion**. Based on the nature of the two assays, growth promotion could only be detected by the CFS broth assay. Among 31 pairings promoting growth, 9 were strong (++) and 22 were weak (+) (Table 3). *Pseudomonas* spp. and *Enterobacteriaceae* were the most common growth-promoting effector isolates; less-common effectors included *Bacillus* sp. strains A30g and E0g, *Yersinia frederiksenii* A8h, and *L. mesenteroides* B30b (Table 1 and Fig. 3C). The isolates stimulating the strongest growth promotion effects were *Bacillus* sp. strains A30g and E0g, and *Serratia* sp. isolates C0b, C30b, E8c, E8i, and E30j. The targets most strongly promoted were *Pseudomonas* sp. isolates D0g and D8g, *B. thermosphacta* A0b, *C. maltaromaticum* D8c, *Leuconostoc carnosum* F30j, and *L. mesenteroides* (Fig. 3C).

Although most growth-promoting activity reduced DT and/or increased GR (Table 4), MPD was enhanced in some interactions. For example, *Bacillus subtilis* E0g increased the MPD of *Pseudomonas* sp. D8g by 0.45 $OD_{540}$ units. Similarly, *Serratia* sp. E8c increased the MPD of *Pseudomonas* sp. D0g by 0.35 $OD_{540}$ units (Table A1: Appendix A).

**Effector species**. Results of spot-lawn and CFS assays showed isolates inhibiting more than 10 targets predominantly belonged to the genera *Pseudomonas*, *Bacillus* and *Carnobacterium* (Table 1; Fig. 3A and B). All six *Pseudomonas* effector isolates, except B0i,

inhibited at least nine targets, with *Pseudomonas* sp. D0b inhibiting 18 targets (Table 1). *Pseudomonas* sp. B0i had a more limited spectrum, inhibiting only six targets. *Bacillus* sp. A30g and E0g each inhibited 16 targets. *Carnobacterium maltaromaticum* inhibited 5 (C0a) to 10 (C8h) targets. *Carnobacterium* F8g, not identified by 16s rRNA sequencing at the species level, inhibited seven targets, and *Carnobacterium divergens* three to eight targets. *Staphylococcus epidermidis,* represented by one isolate (F30c), inhibited four targets. Live effector cells of the family *Enterobacteriaceae,* including *Hafnia alvei, Serratia* spp., and *R. aquatilis*, produced lower levels of inhibition against a small number of targets on spot lawns and against an even smaller group of targets in the CFS assay (Fig. 3A and B). No inhibition by *H. alvei* E30e was observed in either assay.

Intraspecies inhibition was observed as well. For example, *C. divergens* D30f and *C. maltaromaticum* D8c were inhibited by effector isolates of the same species in both spot-lawn and CFS assay (Fig. 3A and B). Similarly, *L. carnosum* F30d and F30h inhibited *L. carnosum* F30j. Other interesting observations included effectors inhibiting targets on agar, but promoting growth of the same target in broth (e.g., *Pseudomonas* sp. E0f as effector and *C. divergens* D30f as target) (Fig. 3).

**Target species.** Based on both assays, the most frequently inhibited species were *C. divergens* D30f, *C. maltaromaticum* D8c, *Pseudomonas sp.* D0g, *S. epidermidis* F30c and *B. thermosphacta* A0b, with 51.3, 48.7, 47.4, 44.7, and 43.6% of effectors inhibiting these isolates, respectively (Table 2). Interestingly, while being the most commonly inhibited species, growth of *C. divergens* D30f and *C. maltaromaticum* D8c was also promoted by the largest number (25.6%) of effector isolates (Table 2).

Growth-promotion was target-dependent and restricted to a relatively small number of isolates, i.e., *Carnobacterium* sp. strains D30f and D8c, *Pseudomonas* sp. strains D8g and

D0g, *Bacillus* sp. A30g, and *B. thermosphacta* A0b (Table 2 and Fig. 3C). Among nine interactions showing strong growth promotion, five targets were *Pseudomonas* spp. (Fig. 3C). Both *Bacillus* sp. strains A30g and E0g promoted the growth of *Pseudomonas* sp. D8g, displaying PS of 0.15 and 0.32, respectively (Table A1: Appendix A). *Serratia* sp. E8c promoted the growth of both *Pseudomonas* sp. D8g and D0g at PS of 0.37 and 0.12, respectively (Table A1: Appendix A).

**Interactions measured by spot-lawn versus CFS-broth assay.** *Pseudomonas* isolates inhibited more targets on agar (3 to 18 isolates) than in broth (1 to 4 isolates) (Table 1and Fig. 3A and B). The influence of test method was especially evident for *Pseudomonas* sp. D0b, which inhibited only one target in broth but inhibited 18 on agar. *Pseudomonas* isolates were often associated with a diffuse inhibition zone (Fig. 3A). Specifically, diffuse zones were observed for thirteen, nine and eight targets by *Pseudomonas* sp. strains D0b, F0b, and D0g, respectively.

Likewise, *Bacillus* sp. A30g inhibited 14 targets on agar versus seven in broth. *Bacillus subtilis* E0g, however, inhibited the same number of targets by both assays. *C. maltaromaticum* effectors inhibited a wider range of target strains/species in broth compared to agar (Fig. 3A and B). For example, *C. maltaromaticum* C30h inhibited nine of 20 targets in broth, but only three on agar (Fig. 3A and B and Table 1). Overall, by broth assay, Gram-positive bacteria inhibited more target bacteria and displayed relatively stronger inhibition strength compared to Gram-negative bacteria (Fig. 3B). However, no significant difference between these two groups was observed by agar assay (data not shown).

## DISCUSSION

In food, bacterial strains rarely exist in isolation (Faust and Raes, 2012) but rather as members of a microbial community influencing food product quality and shelf-life. The structure of this community is not only affected by intrinsic and extrinsic environmental factors but also possibly by interactions among specific bacteria (Faust and Raes, 2012; Perez-Gutierrez et al., 2013; Wolfe et al., 2014), influencing food quality and safety.

In the present study, we report numerous interactions, tested by both agar- and broth-based assays, among a large and diverse group of bacteria isolated from commercial Australian VP beef (Fig. 3). Among the 39 effector and 20 target isolates tested, representing a total of 774 pair-wise tests, 28.6% (221 pairings) showed inhibition and 4.2% (31 pairings) promotion of target growth.

These studies were conducted in bacteriological media, and under an aerobic atmosphere at 25°C. Although it may be argued that bacterial densities tested in these studies were high, such concentrations and cell-cell proximities may exist in food microenvironments, since bacteria are known to preferentially bind and colonize to specific structures (Zulfakar et al., 2012). While the interpretation of these studies is limited to these specific conditions, they offer insight into potential inter-isolate interactions occurring before and shortly after beef primals are vacuum-packaged. Additional studies are underway to quantify interactions under conditions more relevant to long-term refrigerated storage of refrigerated VP beef.

LAB have been extensively studied as protective cultures for extending food shelf-life and enhancing food safety. They inhibit growth of some spoilage and pathogenic bacteria, such as *Carnobacterium* spp., *B. thermosphacta, Listeria* spp., *Salmonella* spp., and *Staphylococcus aureus*, through the action of bacteriocins, organic acids and/or other antibacterial substances (Cotter et al., 2013; Li et al., 2011; Martin-Visscher et al., 2008). In the present study, *C.*

*maltaromaticum* isolates inhibited from five (C0a) to ten (D0h) target isolates (Table 1). In contrast, other LAB species did not display as large an inhibition spectrum as C. *maltaromaticum*; for example, most *C. divergens* inhibited no more than five targets, whereas *L. carnosum* inhibited two (Table 1). Interestingly, *C. maltaromaticum* and *C. divergens* also showed strong intraspecies inhibition (Fig. 3A and B), an observation consistent with the studies of Martin-Visscher et al. (2008) and Worobo et al. (1995). As such, *C. maltaromaticum*, and to a lesser extent *C. divergens*, may have a strong influence on bacterial community structure in VP beef.

The inhibition spectrum of most LAB measured by the agar spot-lawn assay was not as diverse as that by CFS assay, for example, *C. maltaromaticum* D0h (Fig. 3), whereas in broth, extended DT and decreased GR were more frequently observed than decreased MPD (Table A1: Appendix A). These differences may due to inhibitory factors in CFS, such as disassociated lactic acid and bacteriocins, commonly produced by *Carnobacterium* spp.(Bali et al., 2014).

When considering the combined results of spot-lawn and CFS assays, *Pseudomonas* spp., with the exception of effector *Pseudomonas* sp. B0i, displayed high antagonistic behaviour, inhibiting, on average, almost half of the targets (Fig. 3A and B and Table 1). *Pseudomonas* sp. D0b inhibited 18 of the 20 targets (Table 1). Similarly, Aguirre-von-Wobeser et al. (2014), using the spot-lawn method, also found *Pseudomonas* spp., isolated from an aquatic environment, were the most highly antagonistic strains. Published reports show plant and clinical strains of *Pseudomonas* (e.g., *Pseudomonas putida*, *P. fluorescens*, and other *Pseudomonas* spp.) produce secondary antimicrobial metabolites, including enzymes, volatiles (hydrogen cyanide), cyclic lipopeptides, and antibiotics (Kruijt et al., 2009; Kuiper et al., 2004; Li et al., 2013b). These have been applied in plant pathology to control fungal

pathogens and in clinical studies to inhibit pathogenic strains (Afsharmanesh et al., 2010; Cardozo et al., 2013; Trippe et al., 2013).

However, antibacterial compounds might not explain all the inhibitory activities of *Pseudomonas* spp., since inhibition patterns of *Pseudomonas* spp. differed markedly between spot-lawn and CFS assays. For example, *Pseudomonas* sp. D0b CFS only inhibited one target by CFS, but seventeen by spot-lawn. This may indicate live effector cells, not just CFS, are required for target inhibition, as reported by Russell et al. (2011), who found *Pseudomonas* spp. killed bacteria by exporting functional molecules through the type VI secretion system, a form of contact-mediated killing. It also may suggest physiological responses of *Pseudomonas* spp. differ in solid versus liquid media.

It was also noted that growth of *C. divergens* D30f and *C. maltaromaticum* D8c was promoted by CFS from most *Pseudomonas* isolates, although promotion strength was low. Thus, in the early stages of vacuum-packaging of beef, when oxygen is present, the growth-promoting and/or -inhibiting effects of *Pseudomonas* spp. on sensitive bacteria, such as *Carnobacterium* spp., may influence the levels and composition of bacterial species during later stages of VP storage. Further studies are required to elucidate the underlying interacting mechanism(s).

Both *Bacillus* sp. strains E0g and A30g influenced the growth of a wide spectrum of isolates, inhibiting 16 of 20 targets. Members of this genus are known to produce antimicrobial compounds (Teixeira et al., 2013). Baindara et al. (2013) characterized two antimicrobial peptides produced by a *B. subtilis* strain, which showed antagonistic properties against Gram-positive bacteria, including *S. aureus* and *Listeria monocytogenes*. Other *Bacillus* species have been reported to produce bacteriocins and biosurfactants (Singh et al., 2012; Velho et al., 2013); the bacteriocins inhibited the growth of a large range of Gram-

positive and Gram-negative bacteria. *Bacillus subtilis* E0g strongly inhibited most Gram-positive targets, including *C. maltaromaticum* D8c, *B. thermosphacta* A0b, *Bacillus* sp. A30g, *S. epidermidis* F30c, *L. carnosum* F30j, and also some Gram-negative species, such as *Serratia* spp. and *Pseudomonas* spp. (Fig. 3). Unlike *B. subtilis* E0g, *Bacillus* sp. A30g only displayed a wide inhibition spectrum when tested by spot-lawn assay. This indicates inhibition by *Bacillus* sp. A30g may be contact-dependent (Dubey and Ben-Yehuda, 2011).

*Enterobacteriaceae*, such as *H. alvei*, *Serratia* spp., and *R. aquatilis*, produced a relatively lower level of inhibition under the test conditions (Fig. 3A and B). *Staphylococcus* spp. were studied by Cogen et al. (2010) and were shown to possess antimicrobial activity against skin pathogens such as *S. aureus* via phenol-soluble modulins. Nevertheless, to our knowledge, *S. aureus* has not been well studied for antimicrobial properties in food. The mechanism(s) of *S. epidermidis* F30c inhibition requires further study.

By broth assay, the growth of target isolates was promoted in 4.2% of the effector and target combinations. Most effector isolates (84%) enhancing target growth were Gram-negative bacteria, including *Pseudomonas* spp. and members of the *Enterobacteriaceae*, in addition to three other isolates (*L. mesenteroides* B30b, *Bacillus* sp. A30g and *B. subtilis* E0g (Fig. 3C). Growth promotion also appeared to be target-dependent, centering on a small range of targets, namely, *Pseudomonas* sp. D8g, *B. thermosphacta* A0b, *C. maltaromaticum D30f, C. divergens* D8c and *L. carnosum* F30j. A review of the literature shows promotion of bacterial growth by effector isolates has been less frequently reported compared to inhibition. Possible reasons include the spot-lawn method, a test format not readily detecting growth-promotion, being a primary method used in many previous studies (Aguirre-von-Wobeser et al., 2014; Lo Giudice et al., 2007; Perez-Gutierrez et al., 2013), and that primary interests of applied food microbiology are in extending shelf-life and food quality.

The growth of two *Carnobacterium* spp. isolates was enhanced by a large number of effector isolates, including *Serratia* spp. and *Pseudomonas* spp. (Fig. 3C). As mentioned earlier, *Carnobacterium* spp. also inhibited a large spectrum of targets. These combined observations, as well as *Carnobacterium* spp. being a facultative anaerobe, may result in this genus being more dominant in meats stored under VP conditions (Casaburi et al., 2011; Kiermeier et al., 2013).

In the present study, *Leuconostoc* sp. F30e failed to grow in BHI at 25$^{o}$C, and thus influences on the growth of this strain were not measured by CFS-broth assay. According to other studies, some *Leuconostoc* species, such as *Leuconostoc gelidum*, are isolated form chill-stored foods and may not readily grow at elevated temperature, including 25$^{o}$C used here (Cai et al., 1998; Kim et al., 2000; Shaw and Harding, 1989).

While our general focus was to measure growth inhibition and promotion, we observed different inhibition zone morphologies on agar, possibly indicating different mechanisms of action. Undefined (diffuse) inhibition zones have been observed in antibiotic resistance studies (Deshpande et al., 2002; Steward et al., 2005), and interpreted as low levels of bacterial resistance. We noted that *Pseudomonas* spp. often produced such a diffuse type of inhibition zone.

We measured microbial interactions among bacteria isolated from Australian VP beef, which may, in part, help explain the succession of bacterial communities. However, direct translation of these results to actual bacterial community formation in beef environments must consider that studies used bacteriological broth, relatively high densities of cells, and pair-wise comparison of isolates (Wolfe et al., 2014).

# Chapter 4

# General characterization of mechanisms mediating bacterial interactions on vacuum-packaged beef


## ABSTRACT

In Chapter 1, a large number of bacterial interactions were identified among bacteria isolated from vacuum-packaged beef. Elucidation of mechanisms mediating these interactions is important to further understand the effect of environmental factors, and the role of these interactions, in shaping diverse bacterial communities on VP beef. This study characterized factors influencing interactions between eight combinations of effector and target beef isolates, which previously showed strong growth inhibition or promotion. Results demonstrated the inhibitory effect of two isolates of *Carnobacterium maltaromaticum* and one isolate of *Bacillus subtilis* was mediated by heat- and pH-stable proteinaceous substances. In contrast, the inhibitory effect of three isolates of *Bacillus* sp., *Pseudomonas putida*, and *Pseudomonas* sp., on corresponding isolates of *Yersinia enterocolitica*, *C. maltaromaticum*, and *B. subtili*s, occurred only in the presence of live effector cells, yet was not contact-dependent. Compounds produced by *B. subtilis* and *Serratia* sp. that promoted the growth of *P. lundensis* were non-proteinaceous, and heat- and pH-stable. This study characterized factors mediating growth inhibition and promotion, and showed bacterial interactions were mediated by diverse mechanisms.

## INTRODUCTION

Bacteria rarely exist in isolation, but as members of communities in any given niche, in which they compete or cooperate within the community (D'Onofrio et al., 2010; Faust and Raes, 2012; Lo Giudice et al., 2007). Two forms of competition have been realized in microbial ecology studies: 1) interference competition in which one bacterium directly impairs another; and 2) exploitative competition where community members compete for nutrients and space (Cornforth and Foster, 2013; Hibbing et al., 2010).

A large number of bacterial species produce defence compounds such as organic acids, bacteriocins, antibiotics and hydrogen peroxide. Quorum molecules and contact-dependent communication are also involved in these contests (Aoki et al., 2005; Cotter et al., 2013; De Keersmaecker et al., 2006; Dubey and Ben-Yehuda, 2011; Gobbetti et al., 2007; Russell et al., 2011; Skandamis and Nychas, 2012). Cooperation between bacteria is frequently observed, as well (Faust and Raes, 2012). For example, D'Onofrio et al. (2010) found the growth of uncultured bacteria was promoted by siderophores produced by adjacent organisms from marine sediment.

Since refrigerated vacuum-packed meat environments contain a rich supply of nutrients, diverse bacterial species are present at the time meat is packaged (Ercolini et al., 2011). Over time, fewer species dominate the bacterial community due to selective environmental pressures. For example, lactic acid bacteria (LAB), *Enterobacteriaceae*, and *Brochothrix thermosphacta* tend to predominate in vacuum-packaged beef (Ercolini et al., 2011; Youssef et al., 2014a). Bacterial interactions may also be additional forces shaping the bacterial community. For instance, LAB have been shown to decrease the growth of *B. thermosphacta* (Russo et al., 2006). Furthermore, quorum sensing compounds extracted from meat increased the growth rate of *Pseudomonas fluorescens* and *Serratia marcescens* (Nychas et al., 2009).

In Chapter 1 (Zhang et al., 2015), a number of growth- inhibition and -promotion interactions were found between dominant bacteria isolated from Australian vacuum-packaged beef. *Carnobacterium*, *Pseudomonas*, and *Bacillus* were the main genera displaying strong inhibitory activity. A number of effectors inhibited targets by both agar assay (live cells of effector and target) and cell-free supernatant (CFS)-broth (effector CFS plus live target cells) assays. Examples include *C. maltaromatcium* B0f and D0h inhibiting *C. maltaromatcium* D8c, and *B. subtilis* E0g inhibiting *B. thermosphacta* A0b. However, a number of other inhibitions only occurred with live effector cells, such as *Bacillus* sp. A30g versus *Y. enterocolitica* B8b, *P. putida* D0b versus *C. maltaromaticum* D8c, and *Pseudomonas* sp. D0g versus *B. subtilis* B30a. These observations indicate different mechanisms of inhibition. Similarly, different mechanisms may be involved in instances of target growth-promotion, such as where both *B. subtilis* E0g and *Serratia* sp. E8c strongly promoted the growth of *P. lundensis* D8g.

This Chapter describes general characterisation of mechanisms responsible for growth-promoting and -inhibiting activity for eight combinations of effector and target isolates, displaying various interaction styles and relative strong interaction strength (Zhang et al., 2015). This information aids in selecting strains for growth studies described in Chapters 3 and 4, and towards quantifying the effect of environmental factors on bacterial interactions in beef bacterial communities.

## MATERIALS AND METHODS

**Bacterial isolates.** Eight combinations of effector and target isolates, which displayed diverse interaction style and relatively strong growth inhibition or promotion, were chosen for study ((Zhang et al., 2015); Table 1). The effectors included *Carnobacterium*, *Bacillus*,

*Pseudomonas*, and *Serratia*. Targets included *Carnobacterium*, *Brochothrix*, *Yersinia*, *Bacillus*, and *Pseudomonas*.

**TABLE 1** Eight combinations of effector and target isolates and the corresponding interaction strength (Zhang et al., 2015).

| | Effector | Target | Interaction strength[a] | |
|---|---|---|---|---|
| | | | Live cells (Agar, mm) | CFS (Broth) |
| Inhibition | *Carnobacterium maltaromaticum* B0f | *Carnobacterium maltaromaticum* D8c | 4.48 | 1 |
| | *Carnobacterium* | *Carnobacterium* | 4.82 | 1 |
| | *Bacillus subtilis* E0g | *Brochothrix thermosphacta* | 1.49 | 1 |
| | *Bacillus* sp. A30g | *Yersinia* | 2.65 | 0[b] |
| | *Pseudomonas putida* | *Carnobacterium* | 6.04 | 0 |
| | *Pseudomonas* sp. D0g | *Bacillus subtilis* B30a | 2.30 | 0 |
| Promotion | *Bacillus subtilis* E0g | *Pseudomonas lundensis* D8g | /[c] | 0.15 |
| | *Serratia* sp. E8c | *P. lundensis* D8g | / | 0.37 |

[a] Interaction strength was designated as diameter of inhibition zone in agar assay, and relative inhibition strength which was calculated using detection time, growth rate and maximum population density in cell free supernatant (CFS) broth assay. The value was generated from the average data of triplicates of one experiment for spot-lawn assay and of duplicates of one experiment for CFS assay, respectively (Zhang et al., 2015).

[b] No interaction was observed.

[c] Agar assay was not able to test growth-promoting activity (Zhang et al., 2015).

**Cell-free supernatant (CFS) preparation, and pH, temperature, and enzyme treatments.**

*Preparation of CFS.* Effector isolates were inoculated into brain heart infusion broth (BHI; Amyl Media Ltd, Australia) from a colony grown on tryptone soy agar (TSA, Oxoid Ltd, Australia). Cultures were grown at 25$^{o}$C for 24 h, and then centrifuged at 10 000 × *g* for 5 min. Supernatant was filtered through a 0.2 µm pore-sized filter (Corning®, Germany).

*Sensitivity of effector CFS to pH*. The pH of overnight unfiltered CFS was measured. CFS was then adjusted to pH 3, 5, 7, 9 and 11 ( ± 0.1) using 1 M NaOH or 1 M HCl, and incubated at 4$^{o}$C for 2 h (Baindara et al., 2013; Martin-Visscher et al., 2008). After incubation, pH was adjusted to the original value (6.2 ± 0.1 for *C. maltaromaticum* B0f, 6.3 ± 0.1 for *C. maltaromaticum* D0h, 6.7 ± 0.1 for *B. subtilis* E0g, and 6.7 ± 0.1 for *Serratia* sp. E8c), and then filtered-sterilised. Since NaCl was formed when CFS pH was adjusted, an equal volume of NaCl was added to un-pH-adjusted CFS (negative control).

*Sensitivity of effector CFS to temperature*. One millilitre of CFS was incubated in a heating block (Bio-strategy, Australia) at 37, 70, and 100$^{o}$C for 30 min, and then cooled to 25$^{o}$C. Controls were CFS incubated at 4$^{o}$C.

*Sensitivity of effector CFS to degradative enzymes*. CFS was treated with 1 mg/ml (final concentration) of lipase, *α*-amylase, catalase, papain, proteinase K, and pronase E (dissolved in $H_2O$; Sigma - Aldrich, Australia), and kept at 37, 25, 25, 25, 37, and 37$^{o}$C according to manufacturer's instructions for 2 h, respectively. Controls were CFS containing the same volume of $H_2O$ for enzyme preparation, and $H_2O$ containing the same final concentration of enzymes.

**Effect of CFS treatment on inhibition/promotion of isolate growth.** After the treatments described above, CFS was tested for inhibition- or promotion- activity by agar overlay or CFS-broth assay.

The inhibitory activity of CFS of *C. maltaromaticum* B0f and D0h against target *C. maltaromaticum* D8c was determined by the agar overlay method, as described by Aween et al. (2012). Briefly, overnight cultures of the target isolate were adjusted to cell density of $10^8$ cfu/ml (an optical density at 600 nm ($OD_{600}$), 0.10 – 0.15) and then diluted to $10^7$ cfu/ml using BHI. Nine millilitres of melted TSA (0.7% agar, g/v; 50$^o$C) mixed with 1 ml of bacterial suspension was poured onto TSA plates. After solidification, 10 µl of CFS prepared as above was spotted onto the agar surface. After incubating at 25$^o$C for 24 h, the inhibition zone was photographed and the inhibition diameter determined using software Image J (version 1.49; [http://rsb.info.nih.gov/ij/index.html]). Effect of CFS treatments was evaluated as the percentage in inhibition reduction, calculated as the difference of inhibition diameter between treated CFS and the corresponding control, divided by the inhibition diameter of the control.

Since the inhibition effect of CFS of *B. subtilis* E0g on the growth of *B. thermosphacta* A0b was weak (Table 1) and not easily observed via agar assay, it was measured by broth assay using a BioscreenC instrument (Growth Curve Ab Ltd, Finland), as described in our previous study (Zhang et al., 2015). Untreated CFS was used as a positive control, and 1 M PBS and BHI (pH of both were 7.4 ± 0.1) as two negative controls. Four replicates (four wells in micro well plates) were used for each treatment, and two trials performed on different days. Since the agar assay did not detect growth-promotion (Zhang et al., 2015), the promoting activity of *B. subtilis* E0g CFS (vs *P. lundensis* D8g) and *Serratia* sp. E8c CFS (vs *P. lundensis* D8g) was determined via broth assay.

For the CFS-broth assay, the time to reach $OD_{600}$ of 0.1 (background corrected data) was recorded as "detection time" (DT). Growth rate (GR, $\log_{10}$(OD)/h) and maximum $OD_{600}$ (the highest $OD_{600}$ a growth curve able to reach) were calculated as described in our previous study (Zhang et al., 2015). Inhibition strength (IS) or promotion strength (PS) (Zhang et al., 2015) was then calculated according to each growth parameter for treated CFS and control. Effect of CFS treatments was evaluated as the percentage in inhibition or promotion activity reduction, calculated as the difference of IS or PS between treated CFS and the corresponding control, divided by the IS or PS of the control.

**Effect of *Bacillus* sp. A30g, *Pseudomonas putida* D0b and *Pseudomonas* sp. D0g live cells on *Pseudomonas lundensis* D8g growth.** The inhibitory activity of live cells of three effector isolates on *P. lundensis* D8g was tested in two formats to determine if cell contact was required.

In the first experiment (Fig. 1A), overnight cultures of effector isolates and corresponding target isolates were adjusted to $10^7$ cfu/ml in BHI. One millilitre of effector isolate suspension or un-inoculated BHI (control) was mixed with 9 ml of melted TSA and poured onto plates. After solidification, a piece of 0.2 µm pore-sized sterile filter paper (Diameter, 25 mm; Nuclepore, Canada) was placed on top of the agar layer. Then, 10 µl of target isolate suspension was spotted on top of the filter. After incubating at 25$^o$C for 12 h, the target bacterial cells were harvested by washing the top of the filter using 1 ml of peptone water (0.1% (g/v) bacteriological peptone, 0.85% (g/v) NaCl, pH 7.3 ± 0.2). The $OD_{600}$ of washing fluid was measured.

In the second experiment (Fig. 1B), a mixture containing 1 ml of target isolate suspension ($10^7$ cfu/ml) and 9 ml of melted TSA was poured onto plates. After solidification, filter paper was placed on top of the agar layer. Ten microliter of effector isolates ($10^8$ cfu/ml) was

spotted onto the top of the filter or directly onto the surface of top agar layer (control). After incubation at 25$^{o}$C for 12 h, the filter paper was removed and the inhibition zone photographed.

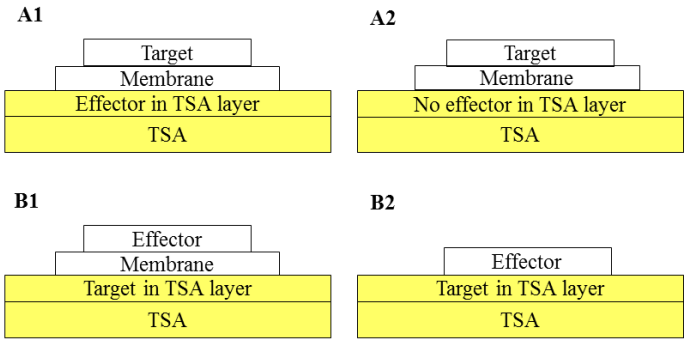



**FIG 1** Layer design of trials separating live cells of effector and target isolates.

**Statistical analysis.** Difference between treatment and control, or treatment groups, was compared using a Student's t-test in Excel®. A *P* value below 0.05 was considered significant.

## RESULTS

**Treatment of *C. maltaromaticum* B0f and D0h CFS and the effect on inhibition of *C. maltaromaticum* D8c.** Temperature, pH, lipase, $\alpha$-amylase, and catalase treatment of *C. maltaromaticum* B0f and D0h CFS did not significantly affect inhibitory activity on *C. maltaromaticum* D8c, as measured by the agar overlay assay (Table 2). In contrast, treatment of D0h and B0f CFS with 1 mg/ml of proteolytic enzymes (papain, proteinase K, and pronase E) removed the inhibitory effects on D8c.

**TABLE 2** Inhibitory activity of *C. maltaromaticum* B0f and D0h CFS after treatments.

| Treatment | | Reduction (%)[a] in inhibitory activity | |
|---|---|---|---|
| | | B0f | D0h |
| pH | 3 | 0.2 ± 0.3 | 2 ± 2.8 |
| | 5 | 0 | 6 ± 8.4 |
| | 7 | 2.9 ± 4.1 | 4.7 ± 6.7 |
| | 9 | 3.1 ± 1.9 | 0.2 ± 0.3 |
| | 11 | 5.3 ± 1.5 | 0.4 ± 3.8 |
| T ($^{o}$C) | 37 | 3.9 ± 4.2 | 0 |
| | 70 | 2.8 ± 4.1 | 2.5 ± 3.6 |
| | 100 | 9.3 ± 5.8 | 10 ± 0.6 |
| Enzymes | Lipase | 0 | 11 ± 5.9 |
| | $\alpha$-Amylase | 2.1 ± 0.6 | 7.8 ± 1.2 |
| | Catalase | 3.5 ± 6.1 | 2.1 ± 3.6 |
| | Papain | 100 | 100 |
| | Proteinase K | 100 | 100 |
| | Pronase E | 100 | 100 |

[a] Effect of a CFS treatment was calculated as the difference of DI between treated CFS and corresponding control, divided by the DI of control, and then multiplied by 100 to get a percent. The data are represented as the mean of two trials ± standard deviation.

**Treatment of *B. subtilis* E0g CFS and the effect on inhibition of *B. thermosphacta* A0b.** Temperature and pH treatments did not reduce the inhibitory activity of *B. subtilis* E0g CFS on *B. thermosphacta* A0b (Table 3 and Fig. 2). Similarly, inhibition activity was not affected by catalase, lipase, $\alpha$-amylase, or papain treatments. However, inhibitory activity was reduced 100% after treatment with proteinase K and pronase E.

**TABLE 3** The effect of treatments on inhibitory activity of *B. subtilis* E0g CFS on *B. thermosphacta* A0b.

| Treatment | | Inhibition activity reduction[a] |
|---|---|---|
| pH | 3 | - |
| | 5 | - |
| | 7 | - |
| | 9 | - |
| | 11 | - |
| T ($^{o}$C) | 37 | - |
| | 70 | - |
| | 100 | - |
| Enzymes | Lipase | - |
| | $\alpha$-Amylase | - |
| | Catalase | - |
| | Papain | - |
| | Proteinase K | + |
| | Pronase E | + |

[a] Inhibitory activity reduction was shown as '–' (0% reduction) or '+' (100% reduction) .

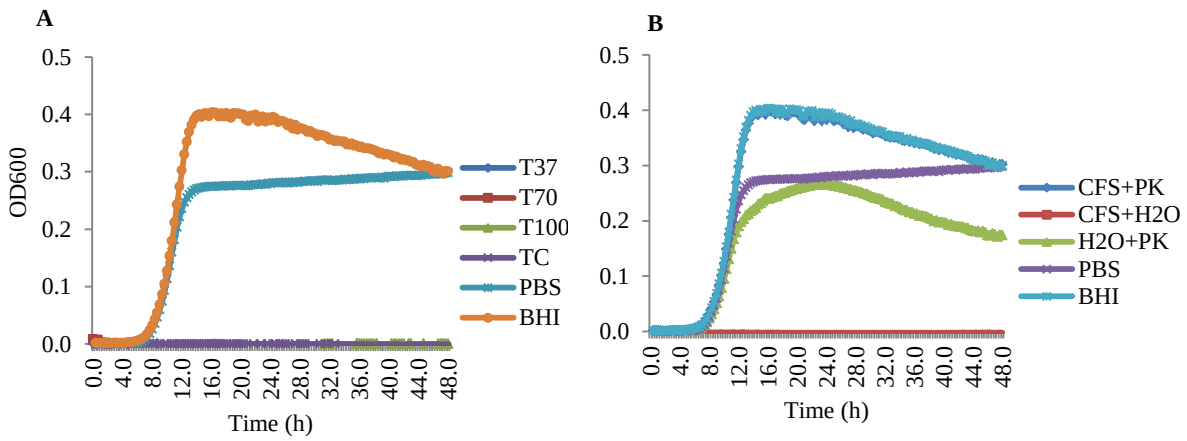



**FIG 2** The effect of temperature (A) and proteinase K (B) on the inhibitory activity of *Bacillus subtillis* E0g CFS on *Brochothrix thermosphacta* A0b.

(A), 0% reduction; T37, T70 and T100 refer to CFS treated at 37°C, 70°C and 100°C, respectively. TC refers to CFS control treated at 4°C.

(B), 100% reduction; CFS+PK, CFS+$H_2O$ and $H_2O$+PK refer to CFS treated with 1mg/ml of proteinase K, CFS added the same volume of $H_2O$, and $H_2O$ added 1mg/ml of proteinase K, respectively.

Both PBS and BHI were used as blank control without adding CFS in panel A and B.

**Measurement of contact-mediated inhibition caused by lives cells of *Bacillus* sp. A30g on *Y. enterocolitica* B8b, *P. putida* D0b on *C. maltaromaticum* D8c, and *Pseudomonas* sp. D0g on *B. subtilis* B30a.** The first test format (Fig. 1A), in which growth of target isolates was measured by enumerating cells on filter paper overlying targets, indicated cell contact was not required to inhibit targets. Specifically, for *Bacillus* sp. A30g vs *Y. enterocolitica* B8b and *P. putida* D0b vs *C. maltaromaticum* D8c, the $OD_{600}$ of target isolates washed from filter papers was 0.24 and 0.112, respectively, significantly lower than controls (0.412 and 0.135) (Fig. 3A). Although $OD_{600}$ of *B. subtilis* B30a was not significantly reduced by live cells of *Pseudomonas* sp. D0g, a reduction in $OD_{600}$ from 0.305 (control) to 0.222 was observed (Fig. 3A).

Similar observations occurred in the second trial, in which inhibition was observed after removal of the 0.2 µm pore-sized filter (Fig. 1B and Fig. 3B). Inhibition was observed for all the three combinations of target and effector isolates.

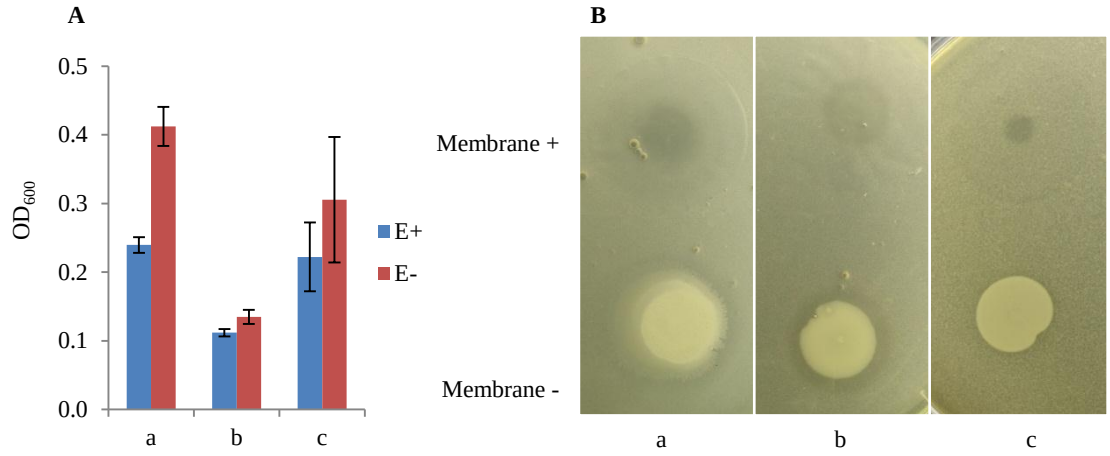



**FIG 3** Characterization of inhibition produced by *Bacillus* sp. A30g, *Pseudomonas putida* D0b and *Pseudomonas* sp. D0g.

Error bars represent x ± standard deviation, and x is the mean of two replicates.

(A) Cell density of target isolate is measured via $OD_{600}$ of washing fluid (Materials and methods). Effector isolates was ('E+') or was not ('E-', control)) added into top agar layer.

(B) 0.2 µm pore-sized filter membrane was ('Membrane+') or was not placed ('Membrane-') to separate live cells of effector and target isolates. Inhibition zone was photographed after peeling off the filter membrane.

a: *Bacillus* sp. A30g (effector) vs *Yersinia enterocolitica* B8b (target)

b: *Pseudomonas putida* D0b (effector) vs *Carnobacterium maltaromaticum* D8c (target)

c: *Pseudomonas* sp. D0g (effector) vs *Bacillus subtilis* B30a (target)

**Treatment of CFS and the effect on growth-promotion of *P. lundensis* D8g by *B. subtilis* E0g and *Serratia* sp. E8c CFS.** The growth-promoting effect of *B. subtilis* E0g CFS

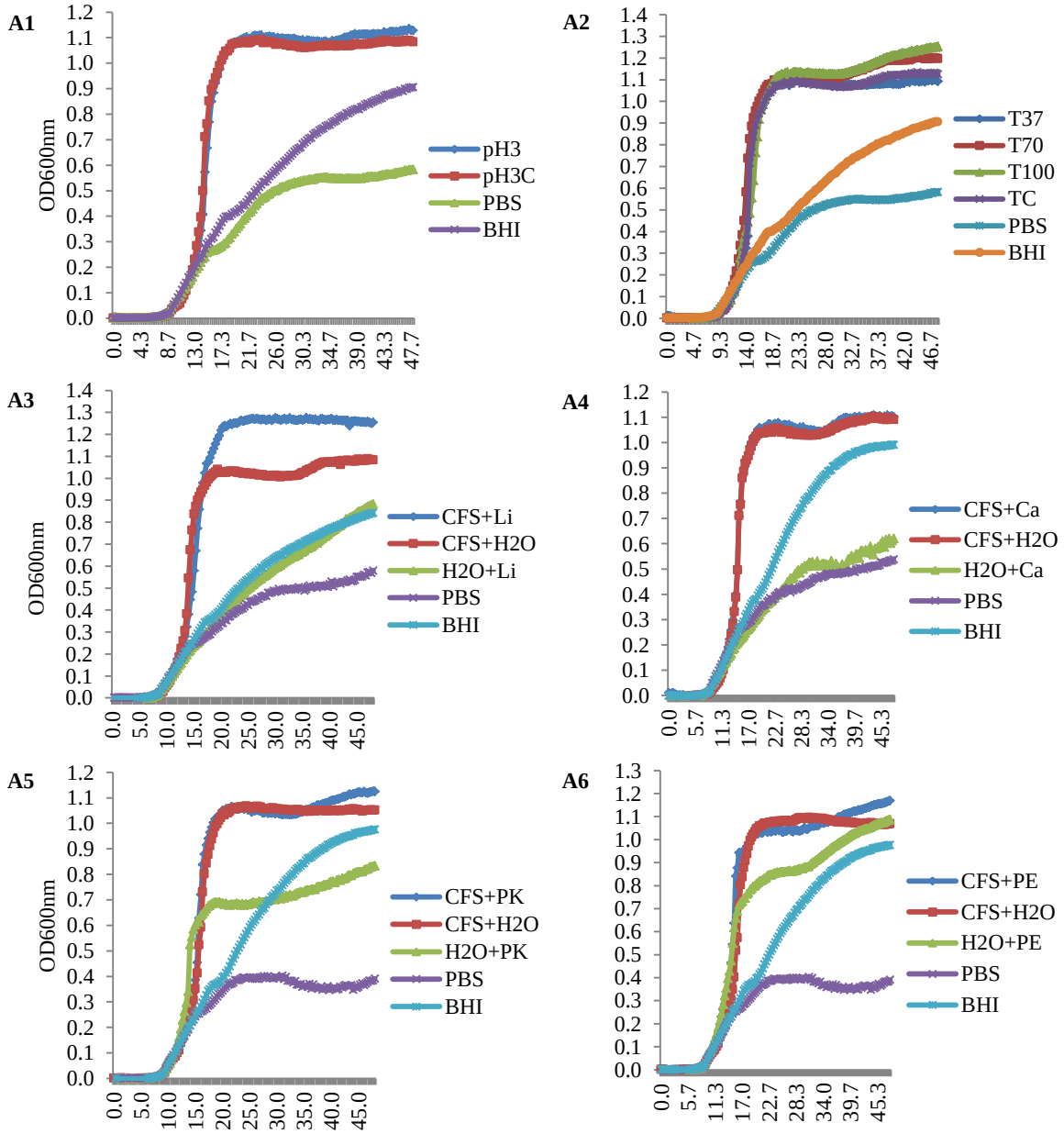
on *Pseudomonas* sp. D8g was not affected by pH, temperature, or enzymes, except for lipase, as tested by broth assay (Table 4 and Fig. 4A). Interestingly, a significantly higher maximum $OD_{600}$ (1.275) for target *P. lundensis* D8g was produced after E0g CFS was treated with 1 mg/ml of lipase, compared to CFS without treatment (1.087) (Fig. 4A3).

The growth-promoting activity of *Serratia* sp. E8c CFS was reduced by pH 3 and 11 treatments, and was increased by lipase, as tested by broth assay (Table 4 and Fig. 4B1 and 3B2). Specifically, GR of target *Pseudomonas* sp. D8g was 0.272 and 0.212 for CFS treated with pH 3 and 11, and 0.327 and 0.328 for controls, respectively. *Serratia* sp. E8c CFS treated with lipase had a higher maximum $OD_{600}$ (1.346 vs 0.973 (control); Fig. 4B4). The growth-promoting activity of E8c CFS effector was not significantly influenced by other treatments.

**TABLE 4** The effect of treatments on promoting activity of *B. subtilis* E0g and *Serratia* sp. E8c CFS on *P. lundensis* D8g.

| Treatment | | Effect[a] | |
|---|---|---|---|
| | | E0g | E8c |
| pH | 3 | - | D[b] |
| | 5 | - | - |
| | 7 | - | - |
| | 9 | - | - |
| | 11 | - | D[c] |
| T ($^{o}$C) | 37 | - | - |
| | 70 | - | - |
| | 100 | - | - |
| Enzymes | Lipase | I[d] | I[e] |
| | α-Amylase | - | - |
| | Catalase | - | - |
| | Papain | - | - |
| | Proteinase K | - | - |
| | Pronase E | - | - |

[a] The effect of treatments on CFS was shown as '–' (0% reduction), 'D' (significant decrease was observed in exponential growth phase, which was 12.6% for 'D[b]' and 55.3% for 'D[c]', respectively), and 'I' (significant increase was observed in stationary phase, which was 76.4 % for 'I[d]' and >100% for 'I[e]', respectively).



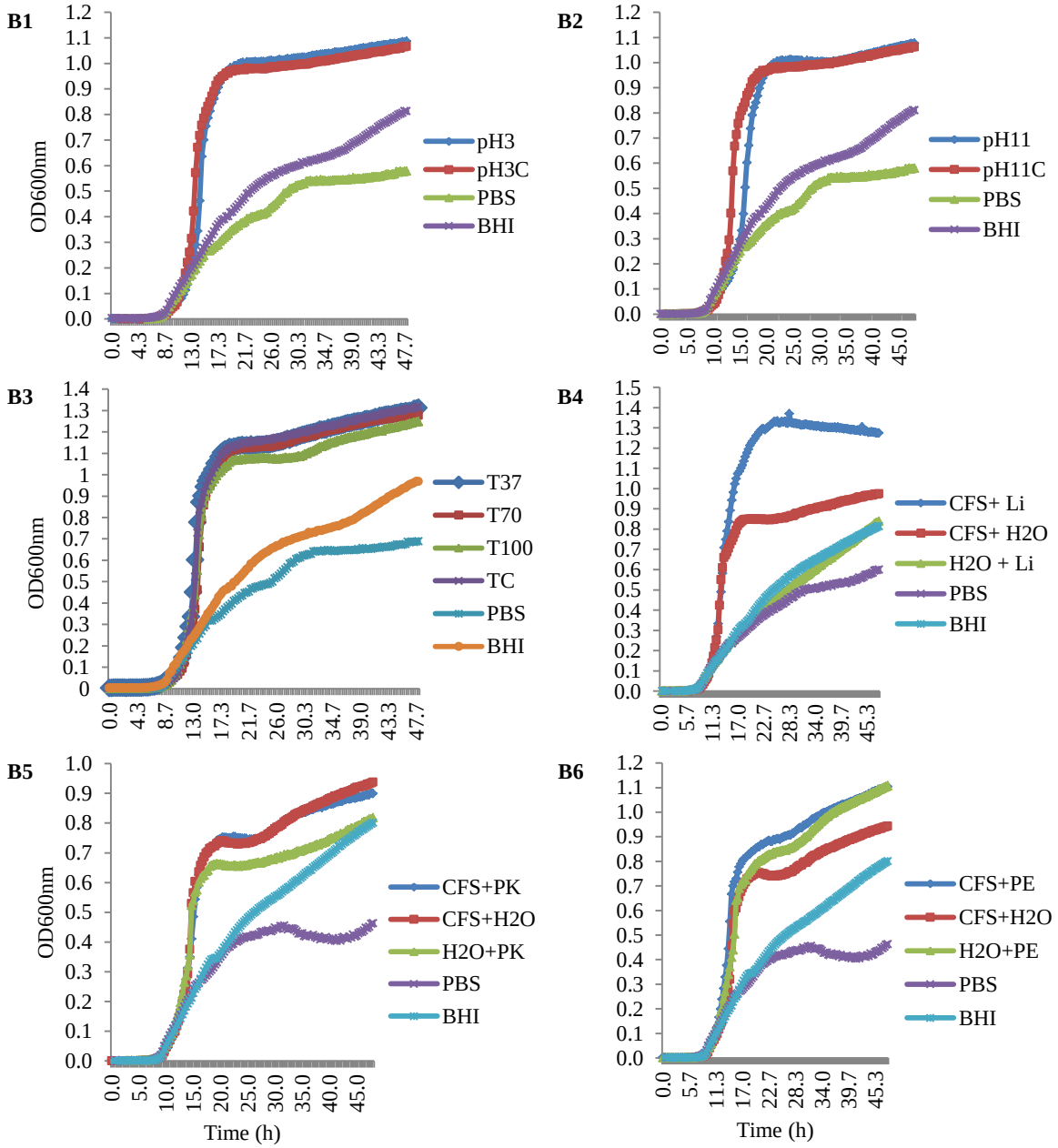



**FIG 4** The effect of treatments on the promoting activity of CFS produced by *Bacillus subtilis* E0g and *Serratia* sp. E8c.

(A1) – (A6), *B. subtilis* E0g (effector) vs *Pseudomonas* sp. D8g (target)

(B1) – (B6), *Serratia* sp. E8c (effector) vs *Pseudomonas* sp. D8g (target)

Both PBS and BHI were used as blank control without adding CFS in graphs A and B.

(A1), (B1) and (B2), pH3 and pH11 refer to corresponding CFS treated with pH 3 and 11, and pH3C and pH11C refer to CFS control for corresponding pH, respectively.

(A2) and (B3), T37, T70 and T100 refer to CFS treated at 37$^{o}$C, 70$^{o}$C and 100$^{o}$C, respectively. TC refers to CFS without heat treatment.

(A3) - (A6) and (B4) - (B6), CFS+Li, CFS+Ca, CFS+PK, CFS+PE refer to corresponding CFS added 1mg/ml of lipase, catalase, proteinase K and pronase E, respectively. CFS+$H_2O$ refer to corresponding CFS added the same volume of $H_2O$. $H_2O$+Li, $H_2O$+Ca, $H_2O$+PK and $H_2O$+PE refer to $H_2O$ added 1mg/ml of lipase, catalase, proteinase K, pronase E, respectively.

## DISCUSSION

### Inhibitory activity of *C. maltaromaticum* B0f and D0h CFS on *C. maltaromaticum* D8c.

*Carnobacterium* is a LAB species that predominates in the bacterial community of vacuum-packaged (VP) beef (Ercolini et al., 2011). Strains of *C. maltaromaticum* produce bacteriocins possessing inhibitory activity, even against isolates of the same species (Martin-Visscher et al., 2008; Tulini et al., 2014). In our previous study, CFS of both *C. maltaromaticum* B0f and D0h were antagonistic against beef bacterial isolates from various species, and produced the largest inhibition against *C. maltaromaticum* D8c (Zhang et al., 2015). Hence, this study used *C. maltaromaticum* D8c as a target isolate to characterise inhibitory activity caused by B0f and D0h CFS.

The inhibitory activities of B0f and D0h CFS were sensitive to proteolytic enzymes including papain, proteinase K and pronase E, evidenced by no inhibition zone on agar plates (Table 2). In contrast, CFS of B0f and D0h retained the majority of inhibitory activity after treatment with the lipolytic enzyme, lipase, the glycolytic enzyme, $\alpha$-amylase, and catalase. This indicates the antibacterial compounds are proteinaceous.

The pH treatment of B0f and D0h CFS, showed CFS was more sensitive to high and low pH, respectively (Table 3). However, on the whole, CFS showed pronounced stability at

100$^{o}$C, and pH from 3 to 11. The data indicate the CFS contains bacteriocin-like substances (Martin-Visscher et al., 2008; Tulini et al., 2014).

Bacteriocins are ribosomally synthesized peptides, which are usually heat-stable and inhibit closely related bacteria (Cotter et al., 2013). *C. maltaromaticum* is known to produce class I and II bacteriocins (Holck et al., 1994; Martin-Visscher et al., 2008; Quadri et al., 1994; Stoffels et al., 1992b; Tulini et al., 2014). Further investigation could clarify the exact type of bacteriocin produced by *C. maltaromaticum* B0f and D0h, although this was not the focus of this thesis.

**Inhibitory activity of *B. subtilis* E0g CFS on *B. thermosphacta* A0b.** CFS of *B. subtilis* E0g was tested by agar assay, displaying a faint inhibition zone. Therefore, the greater sensitivity of the CFS-broth assay was used to measure the effect of pH, temperature and enzyme treatments on CFS.

A large diversity of antibacterial compounds is produced by *Bacillus* spp., including various classes of bacteriocins and surface-active lipopeptides (Baindara et al., 2013; Ghribi et al., 2012; Joseph et al., 2013; Phelan et al., 2013; Xin et al., 2015). Due to the complete loss of inhibitory activity of *B. subtilis* E0g CFS after digestion by proteolytic enzymes, including proteinase K and pronase E, the inhibitory compounds are likely proteins (Table 3). However, the activity was not affected by papain. Similar discrepancies have been reported by Phelan et al. (2013), who found a *B. subtilis* strain produced a class I bacteriocin, subtilomycin resistant to proteinase K but susceptible to a protease mixture (pronase E). *B. subtilis* E0g CFS was not sensitive to lipase, catalase, pH from 3 to 11, and temperatures from 37$^{o}$C to 100$^{o}$C (Table 3). It has been reported that bacteriocins produced by *Bacillus* spp. are thermostable and resistant to a wide range of pH (Baindara et al., 2013; Joseph et al., 2013; Phelan et al., 2013).

In the present study, $\alpha$-amylase alone inhibited the growth of target isolate, *B. thermosphacta* A0b, and therefore the effect of this enzyme on *B. subtilis* E0g CFS could not be tested. Alpha-amylase was previously reported to inhibit the growth of clinical pathogens such as *Legionella pneumophila* (Bortner et al., 1983), *Neisseria gonorrhoeae* (Gregory et al., 1983) and *Porphyromonas gingivalis* (Ochiai et al., 2014), though mechanisms of this inhibition are still unknown.

**Inhibition by live cells of *Bacillus* sp. A30g on *Y. enterocolitica* B8b, *P. putida* D0b CFS on *C. maltaromaticum* D8c, and *Pseudomonas* sp. D0g on *B. subtilis* B30a.** In our previous study (Zhang et al., 2015), inhibitory activity between these three combinations of isolates was observed by agar assay but not observed by CFS-broth assay (Table 1). We hypothesized inhibition would require cell contact between live cells of effector and target isolates.

A 0.2 µm pore-sized membrane was used to separate effector and target live cells. However, the growth of target isolates *Y. enterocolitica* B8b, *C. maltaromaticum* D8c, and *B. subtilis* B30a remained inhibited by *Bacillus* sp. A30g, *P. putida* D0b, and *Pseudomonas* sp. D0g, respectively. In addition, an inhibition zone was observed after removing the filter (Fig. 3). This indicates inhibition by live effector cells is not contact-dependent.

This phenomenon was possibly due to the production of antibacterial compounds induced by the presence of target live cells. Cornforth and Foster (2013) argue that toxin secretion of a number of species, including *Pseudomonas* and *Bacillus*, are frequently induced by environmental stress signals such as nutrient limitation. Slattery et al. (2001) also found that marine bacterial species, *Streptomyces tenjimariensis* was induced to produce antibiotic in the presence of competing bacteria.

**Growth-promoting activity of *B. subtilis* E0g and *Serratia* sp. E8c CFS on *P. lundensis* D8g.** Mutualistic interactions among bacteria are ubiquitous in nature, which involves various mechanisms (McInerney et al., 2008; Woyke et al., 2006). Bacteria metabolize compounds in a variety of ways, and some produce growth-promoting compounds (Grenier and Mayrand, 1986; Wyss, 1989). In the present study, growth-promoting activity of *B. subtilis* E0g and *Serratia* sp. E8c CFS were not sensitive to papain, proteinase K, pronase E, catalase, $\alpha$-amylase, and were heat-stable (Table 4). The CFS of two effector isolates also produced pronounced pH stability from 3 to 11, except that CFS of *Serratia* sp. E8c was slightly inhibited by treatment at pH 3 and 11 (Fig. 4B1 & 4B2).

These findings are partly consistent with the study of Tanaka et al. (2005), in which growth-promoting factors were heat-stable non-peptides with low molecular weights. Nychas et al. (2009) found that CFS containing quorum sensing (QS) compounds could promote the growth of *P. fluorescens* and *Serratia marcescens*. Although, the mechanism is unclear, it is argued QS might sense cell density of a bacterial population and then cause neighbouring cells to utilize extracellular nutrients by changing gene expression of target bacteria (Dourou et al., 2011).

Siderophores have also been reported to play an important role in promoting growth by assisting neighbouring bacteria acquire environmental iron (D'Onofrio et al., 2010). *P. fluorescens* has been reported to possess 24 putative siderophore receptors, allowing the cell to acquire a range of heterologous siderophores, besides its own (Moon et al., 2008). *P. lundensis* D8g used in this study may have a similar property, as it was promoted by siderophores produced by *B. subtilis* E0g and *Serratia* sp. E8c. However, AI-2 (auto inducer-2, QS factor) could also be a potential factor in promoting activity displayed by these two effector isolates. Autoinducer AI-2 is regarded as a universal language for the communication of both inter-species and intra-species. The growth of both Gram-negative and -positive

bacteria (Federle and Bassler, 2003; Skandamis and Nychas, 2012), and of *Escherichia coli* O157: H7 was promoted by an AI-2 mediated process (Dourou et al., 2011).

An interesting phenomenon is that the maximum $OD_{600}$ of *P. lundensis* D8g was further increased by CFS of the two effector isolates after lipase treatment (1 mg/ml). This infers lipase might digest compounds in effector bacteria CFS, making them more easily utilized by target bacteria.

Due to the complexity of the composition of CFS of bacteria cultures, the chemical structure of the growth-promoting factors in the study could not be clarified. However, the susceptibility of these substances to pH, temperature and enzymes may help explain the effect of environmental factors on bacterial interactions on VP beef.

## CONCLUSIONS

Two isolates of *C. maltaromaticum* and one isolate of *B. subtilis* from VP beef produced proteinaceous antibacterial compounds, which are temperature and pH stable. The inhibitory activity of *Bacillus* sp. A30g and two isolates of *Pseudomonas* against target isolates are not contact-dependent, but instead require the presence of live effector cells. The non-peptide substances produced by *B. subtilis* and *Serratia* sp., which are heat- and pH-stable, promote the growth of *P. lundensis*.

# Chapter 5

# Effect of environmental factors on intraspecific inhibitory activity of *Carnobacterium maltaromaticum* strains isolated from vacuum-packaged refrigerated beef


## ABSTRACT

Antibacterial compound production is a prevalent strategy used in inter- and intra-species competition for limited nutrients. Our previous study found vacuum-packaged (VP) beef isolate *Carnobacterium maltaromaticum* D0h strongly inhibited the growth of *C. maltaromaticum* D8c. However, the influence of environmental factors, relevant to the VP beef environment, on this interaction is unknown. This study investigated the effects of temperature, atmosphere, pH, lactic acid, and glucose on the sensitivity of strain D8c to inhibition by D0h cell-free supernatant (CFS), using an agar model system. D0h CFS was applied to agar containing viable cells of D8c, and then the inhibition zone (DI) measured to evaluate CFS inhibitory activity. The inhibitory activity, shown to be proteinaceous, was greatest at 15$^{o}$C, followed by 7, -1, and 25$^{o}$C, and higher under aerobic than anaerobic conditions. Agar supplemented with lactic acid and glucose increased DI. DI was less at pH 6.5, compared to pH 5.5 and 6. Predictive models were produced to model environmental effects on DI. This study provides a quantitative understanding of intra-species interactions, and helps explain how VP beef related environmental factors affect these interactions.

## INTRODUCTION

Bacteria interact in any given niche, including food environments (Blana and Nychas, 2014; Faust and Raes, 2012; Zhang et al., 2015). They cooperate or compete by secreting metabolites, or via direct cell-cell interactions (Aoki et al., 2005; Blana and Nychas, 2014; Cotter et al., 2013; Dubey and Ben-Yehuda, 2011; Pande et al., 2015; Zhang et al., 2015). Such interactions may be important in influencing the shelf-life of meat products through changes in bacterial community structure (Blana and Nychas, 2014; Perez-Gutierrez et al., 2013; Wolfe et al., 2014).

Production of defence compounds is an important strategy for bacteria to compete within bacterial communities (Cotter et al., 2013; Phelan et al., 2012; Zhang et al., 2015). *Carnobacterium maltaromaticum*, a dominant lactic acid bacteria (LAB) species in vacuum-packaged (VP) meat, has been reported to produce inhibitory compounds, including organic acids and bacteriocins (Laursen et al., 2005; Martin-Visscher et al., 2008; Tulini et al., 2014; Youssef et al., 2014a). In our previous study (Zhang et al., 2015), cell-free supernatant (CFS) of various isolates of this species from Australian VP beef displayed inhibitory activity, and isolate D0h was found to have the widest inhibition spectrum against spoilage bacteria, including *Serratia* spp., *Pseudomonas* spp., *Leuconostoc* spp., and other *Carnobacterium* spp. Strain D0h even inhibited an isolate of the same species, namely *C. maltaromaticum* D8c, at 25$^{o}$C under aerobic conditions on standard laboratory media (Zhang et al., 2015). However, the effect of environmental factors on this inhibitory interaction was unknown.

Such environmental conditions include < 1% v/v oxygen atmosphere, refrigerated temperature, pH 5.0 – 6.0, 10 – 100 mM lactic acid, and approximately 0.01 % (wt/wt) glucose (Frylinck et al., 2013; Jones, 2004; Small et al., 2012). Due to the dominant role of *C. maltaromaticum* in the bacterial community in VP beef (Doulgeraki et al., 2012), a better

understanding of factors affecting bacterial interactions, and community development, could help design processes to maintain and/or improve shelf-life.

To date, numerous models have been developed to predict the effect of food-related environmental factors on growth of bacteria. However, due to a lack of quantitative information about bacterial interactions, these models consider the effect of environments on the growth of one or a few species, not accounting for interactions between species (Posada-Izquierdo et al., 2014; Powell et al., 2015).

This study quantified and modelled the effect of environmental factors—atmosphere, temperature, pH, glucose, and lactic acid—on sensitivity of *C. maltaromaticum* D8c to inhibitory factor(s) produced by *C. maltaromaticum* D0h. These *in vitro* findings provide a more mechanistic understanding of interactions between bacterial isolates under various environmental conditions, with potential applications to commercial beef.

## MATERIALS AND METHODS

**Bacterial isolates and CFS preparation**. Effector and target strains of *C. maltaromaticum*, D0h and D8c, respectively, were isolated from VP beef (Small et al., 2012; Zhang et al., 2015) and stored at -80$^{o}$C. Before each experiment, these isolates were separately inoculated into BHI broth (Amyl Media Ltd, Australia) from a single colony on tryptone soy agar (TSA, Oxoid Ltd, Australia) and incubated at 25$^{o}$C for 24 h. CFS produced by D0h was prepared by centrifuging cultures at 10,000 × *g* for 5 min, followed by filtration through a 0.2 µm pore-sized membrane (Corning®, Germany).

**Measurement of CFS inhibitory activity**. The inhibitory activity of isolate D0h CFS on growth of isolate D8c was determined by the agar overlay method as reported by Aween et al.

(2012), with slight modification. Briefly, early stationary phase cultures of D8c were adjusted to $10^8$ cfu/ml by optical density (OD) measurement in a 96-well plate at 600 nm ($OD_{600}$; SPECTROstar Nano Absorbance Reader, Germany); OD values ranged from 0.10–0.15. Ten millilitres of melted TSA (0.7% agar, g/v; 50°C) was mixed with $10^7$ cfu D8c, and poured into agar plates. After solidification, 10 µl of filter-sterilised CFS was spotted onto the agar surface in triplicate, incubated at 25°C for 24 h, and then the inhibition zone photographed and the diameter of inhibition (DI) measured with ImageJ software (v1.49 [http://rsb.info.nih.gov/ij/index.html]).

**Kinetics of production of *C. maltaromaticum* D0h inhibitory compounds**. To investigate the kinetics of inhibitory compound production, *C. maltaromaticum* D0h was inoculated into BHI at an initial cell density of $10^5$ cfu/ml, and then incubated aerobically at 25°C. Culture $OD_{600}$ was measured every 3 h for the first 9 h, and then at 1.5 h intervals. At each time point, CFS was diluted in two-fold serial increments, and then inhibitory activity determined by the agar overlay assay, as described above.

**Effect of environmental factors on the sensitivity of *C. maltaromaticum* D8c to inhibition by *C. maltaromaticum* D0h CFS**. The sensitivity of target isolate D8c to inhibitory compounds produced by D0h was tested using an agar overlay method. However, rather than TSA applied as above, modified brain heart infusion (mBHI) broth, without glucose, was used as the base medium (AM 11-NG, Amyl Media, Ltd., Australia; mBHI contained 10 g blended peptone no. 1, 5 g sodium chloride, 17.5 g brain heart infusion solid, and 2.5 g di-sodium hydrogen orthophosphate, per litre). Conditions included temperature (-1, 7, 15, and 25°C), atmosphere (aerobic and anaerobic), pH (5.5, 6, and 6.5), lactic acid (0, 25, and 50 mM), and glucose (0, 0.56, and 5.55 mM). A full factorial design (4 × 2 × 3 × 3 × 3) was applied.

Different amounts of L(+) lactic acid (Scharlab, Spain) and G(+) glucose (Sigma, USA) were used to prepare 27 variations of mBHI (Table 1). The pH was adjusted to 5.5, 6, or 6.5 (± 0.1) using 10 M NaOH or 32% (g/v) HCl (Table 1). After adding 15 g/L of agar, mBHI medium was autoclaved (121$^{o}$C, 15 min), and pH adjusted when the medium cooled to approximately 60$^{o}$C. The concentration of undissociated lactic acid (UndisLA) was calculated according to the total concentration of lactic acid and final pH.

$$[UndisLA] = \frac{[LA]}{1+ 10^{(pH-3.86)}} \quad (1)$$

Ten millilitres of 50$^{o}$C mBHI mixed with $10^7$ cfu D8c were added to Petri dishes, previously prepared with a bottom layer of 15 ml mBHI agar.

Preliminary experiments showed *C. maltaromaticum* D0h broth cultures contained the highest levels of inhibitory compounds (1600 AU/ml) between 19.5–22.5 h of incubation (Fig. 1). Using CFS from this time interval, three 10 µl aliquots of CFS were added to the agar surface. After CFS was completely absorbed, agar plates were incubated at different temperatures, both aerobically and anaerobically, until inhibition zones were observed (Table 1 and 2). Anaerobic conditions (< 1.0% $O_2$, ≥13% $CO_2$) were created by a GasPak EZ anaerobic pouch (BD, Australia) placed in a sealed jar. Incubation temperature was recorded using data loggers (Thermochron iButton®, Australia). At the end of the experiments, DI was measured.

**TABLE 1** Levels of lactic acid, glucose, and pH for mBHI agar formulations.

| Medium (#) | Lactic acid (mM) | Glucose (mM) | pH | UndisLA (mM)[a] |
|---|---|---|---|---|
| 1 | 0 | 0 | 5.5 | 0 |
| 2 | 0 | 0 | 6 | 0 |
| 3 | 0 | 0 | 6.5 | 0 |
| 4 | 25 | 0 | 5.5 | 0.56 |
| 5 | 25 | 0 | 6 | 0.18 |
| 6 | 25 | 0 | 6.5 | 0.06 |
| 7 | 50 | 0 | 5.5 | 1.12 |
| 8 | 50 | 0 | 6 | 0.36 |
| 9 | 50 | 0 | 6.5 | 0.11 |
| 10 | 0 | 0.56 | 5.5 | 0 |
| 11 | 0 | 0.56 | 6 | 0 |
| 12 | 0 | 0.56 | 6.5 | 0 |
| 13 | 25 | 0.56 | 5.5 | 0.56 |
| 14 | 25 | 0.56 | 6 | 0.18 |
| 15 | 25 | 0.56 | 6.5 | 0.06 |
| 16 | 50 | 0.56 | 5.5 | 1.12 |
| 17 | 50 | 0.56 | 6 | 0.36 |
| 18 | 50 | 0.56 | 6.5 | 0.11 |
| 19 | 0 | 5.55 | 5.5 | 0 |
| 20 | 0 | 5.55 | 6 | 0 |
| 21 | 0 | 5.55 | 6.5 | 0 |
| 22 | 25 | 5.55 | 5.5 | 0.56 |
| 23 | 25 | 5.55 | 6 | 0.18 |
| 24 | 25 | 5.55 | 6.5 | 0.06 |
| 25 | 50 | 5.55 | 5.5 | 1.12 |
| 26 | 50 | 5.55 | 6 | 0.36 |
| 27 | 50 | 5.55 | 6.5 | 0.11 |

[a] UndisLA, the undissociated form of lactic acid, the concentration of which is calculated according to the concentration of lactic acid and pH of corresponding medium ( $[\text{UndisLA}] = [\text{Lactic acid}]/(10^{(pH-3.86)}+1)$ ).

**TABLE 2** Incubation conditions for samples.

| Temperature (°C) | Atmosphere | | Medium #[a] | Incubation time (d) |
|---|---|---|---|---|
| | Aerobic | Anaerobic | | |
| 25 | + | + | 1–27 | 10 |
| 15 | + | + | 1–27 | 19 |
| 7 | + | + | 1–27 | 45 |
| -1 | + | + | 1–3, 5, 6, 8–12, 14, 15, 17–21, 23, 24, 26, 27 | 145 |
| -1 | + | + | 13 | 263 |
| -1 | | + | 4, 16, 22 | 263 |
| -1 | + | + | 7, 25 | NG[b] |
| -1 | + | | 4, 16, 22 | NG |

[a] The media # (no.) are described in Table 1.

[b] NG, no visible growth of target bacteria was observed; diameter of inhibition zone was not measured for these mBHI media.

**Data analysis and model development**. The overall effect of environmental factors on DI was evaluated using analysis of variance (ANOVA), employing the GLM (general linear model) procedure in SAS (v9.3; SAS, Inc., Rockville, MD). If the *P* value from the F-test was below 0.05, a Student *t* test was then performed to identify the significant ($P < 0.05$) pairwise differences. The correlation coefficient between undissociated lactic acid and DI was calculated using Excel® (v2010; Microsoft Corp).

According to the effects of environmental factors investigated with the levels used in this study (i.e., four levels for temperature and three levels each for pH, glucose and lactic acid), a model of DI was developed to predict the values produced under the conditions which were

not explicitly tested in this study (interpolation). The model incorporated the factors which proved to be significant in affecting the inhibitory activity of *C. maltaromaticum*. Separate formulae were derived for aerobic and anaerobic conditions, respectively, using the REG procedure (a general-purpose procedure for regression) with the stepwise selection method in SAS. Variables meeting the 0.05 significance level for entry were included in the model.

**Characterization of antibacterial compounds in D0h CFS**. To characterize the nature of antibacterial compounds, the sensitivity of D0h CFS to pH, temperature, and enzyme treatments was tested.

*pH*. The pH of unfiltered *C. maltaromaticum* D0h culture medium was measured, and then CFS adjusted to pH 3, 5, 7, 9, and 11 (± 0.1) (Table 3) using 1 M NaOH or 1 M HCl, and incubated at 4$^{o}$C for 2 h (Martin-Visscher et al., 2008). After incubation, pH was adjusted to the original value (6.3 ± 0.1), and then filter-sterilised. Since NaCl was formed when CFS pH was adjusted, an equal volume of NaCl was added to unadjusted CFS (negative control).

*Temperature*. One millilitre of filter-sterilised D0h CFS was incubated in a heating block (Bio-Strategy, Australia) at 37, 70, and 100$^{o}$C for 30 min (Table 3), and then cooled to 25$^{o}$C. Control CFS was incubated at 4$^{o}$C.

*Enzyme*. CFS was treated with 1 mg/ml lipase, $\alpha$-amylase, catalase, papain, proteinase K, and pronase E (dissolved in $H_2O$; Sigma-Aldrich, Australia), and incubated at 37, 25, 25, 25, 37, and 37$^{o}$C, respectively, for 2 h, according to manufacturer's instructions (Table 3). The same volume of $H_2O$ was added to CFS for the negative control.

The inhibitory activity of CFS (with or without treatment) was determined by agar overlay assay as described in Section *Measurement of CFS inhibitory activity.*

**TABLE 3** Inhibitory activity of *C. maltaromaticum* D0h CFS after treatments.

| Treatment | | Reduction (%)[a] in inhibitory activity |
|---|---|---|
| pH | 3 | 2 ± 2.8 |
| | 5 | 6 ± 8.4 |
| | 7 | 4.7 ± 6.7 |
| | 9 | 0.2 ± 0.3 |
| | 11 | 0.4 ± 3.8 |
| T ($^{o}$C) | 37 | 0 |
| | 70 | 2.5 ± 3.6 |
| | 100 | 10 ± 0.6 |
| Enzymes | Lipase | 11 ± 5.9 |
| | α-Amylase | 7.8 ± 1.2 |
| | Catalase | 2.1 ± 3.6 |
| | Papain | 100 |
| | Proteinase K | 100 |
| | Pronase E | 100 |

[a] Effect of a CFS treatment was calculated as the difference of DI between treated CFS and corresponding control, divided by the DI of control, and then multiplied by 100 to get a percent. The data are represented as the mean of two trials ± standard deviation.

## RESULTS

**Kinetics of inhibitory compound production by D0h**. Detectable levels of D0h inhibitory compounds occurred between 6 and 9 h of incubation (Fig. 1). Levels increased dramatically during the exponential growth phase, peaking (DI, 14.5mm) at 19.5 h, i.e. end of the exponential phase. There was no significant change ($P > 0.05$) in DI from 19.5 to 22.5 h.

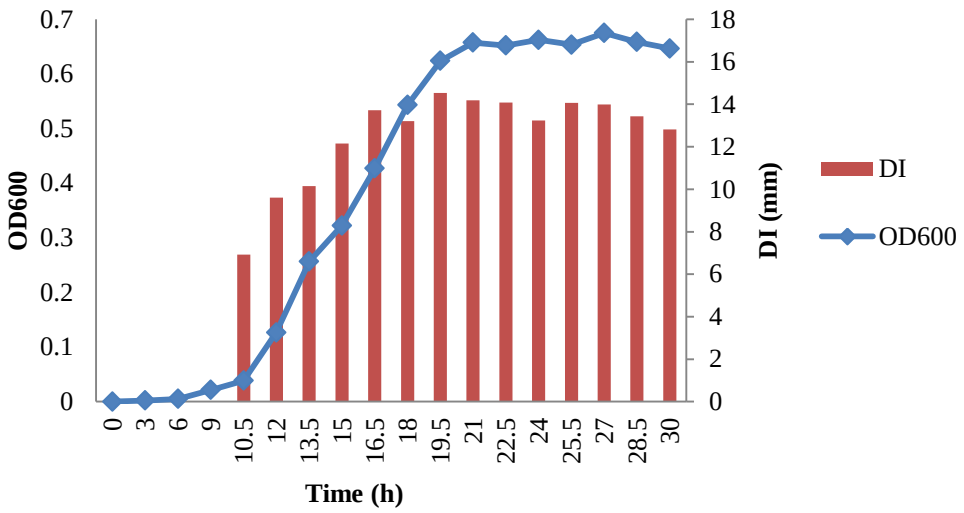



**FIG 1** Kinetics of inhibitory compound production by *C. maltaromaticum* D0h. DI, diameter of inhibition zone.

**Influence of environmental factors on D8c sensitivity to CFS**. Each environmental factor had a significant effect on DI (Fig. 2). For temperature, DI was 13.8 mm at 25°C, significantly less than 20.1, 15.7, and 14.4 mm observed at 15, 7, and -1°C, respectively (Fig. 2A). Overall, aerobic atmosphere produced a larger DI (16.5 mm) compared to anaerobic atmosphere (15.7 mm) (Fig. 2B). Glucose and lactic acid both increased DI significantly; for example, DI was 15.1 mm with no added glucose, but 17.4 mm with 5.55 mM added glucose (Fig. 2C). Lactic acid had a DI of 15.4 mm at 0 mM, increasing to 16.6 mm at 50 mM (Fig. 2D). For pH, DI increased at lower pH, i.e. 15.4 mm at pH 6.5, 15.9 mm at pH 6, and 16.9 mm at pH 5.5 (Fig. 2E).

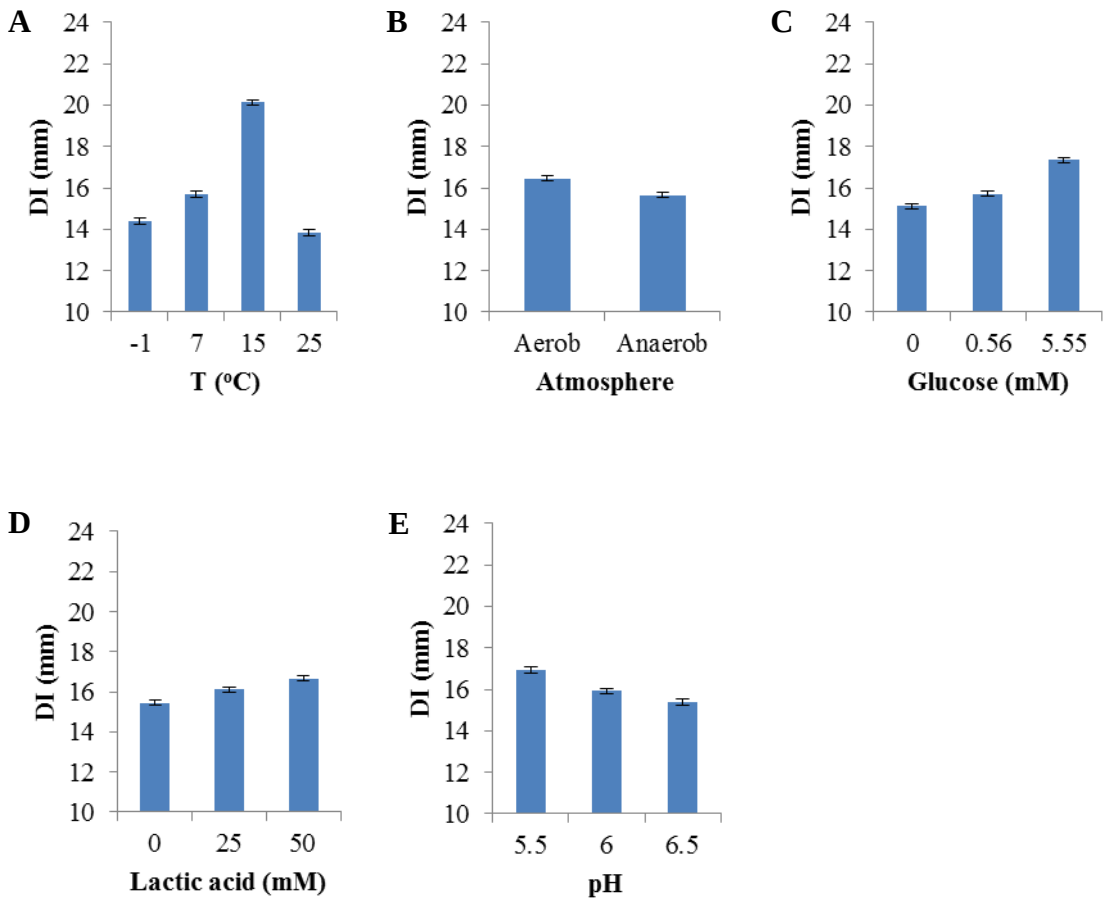



**FIG 2** Effect of environmental factors on diameter of inhibition zone (DI).

Each factor had a significant (F-test, $P < 0.05$) effect on DI. Error bars represent standard error of the mean.

**Interactions between environmental factors on D8c sensitivity to CFS**. Significant interactions were observed between temperature and atmosphere, temperature and glucose, temperature and lactic acid, temperature and pH, glucose and atmosphere, glucose and pH, and pH and lactic acid (Fig 3).

Specifically, the effect of atmosphere at lower temperatures, particularly at 7$^{o}$C, was larger compared to higher temperatures (15$^{o}$C and 25$^{o}$C), with aerobic atmosphere resulting in

larger DI than an anaerobic atmosphere (Fig. 3A). In contrast, glucose, lactic acid, and pH produced a greater effect on DI at higher temperature (Fig. 3B–3D). For example, at 25$^{o}$C, 5.55 mM of glucose increased DI by 2.7 mm, from a mean of 12.5 mm (0 mM glucose) to 15.2 mm; however, at -1$^{o}$C, the mean value only increased by 0.6 mm, from 14.1 mm (0 mM glucose) to 14.7 mm (5.55 mM glucose).

At 5.55 mM glucose, the effects of atmosphere and pH on DI were larger than at lower glucose levels (Figs. 3E and 3F, respectively). In addition, low pH levels increased the effect of lactic acid; for example, at pH 5.5, lactic acid increased DI from 15.6 mm (0 mM lactic acid) to 19 mm (50 mM lactic acid) (Fig. 3G). Also, a positive correlation was observed between concentration of undissociated lactic acid and DI, with a corresponding linear regression $R^2$ value of 0.965 (Fig. 3H).

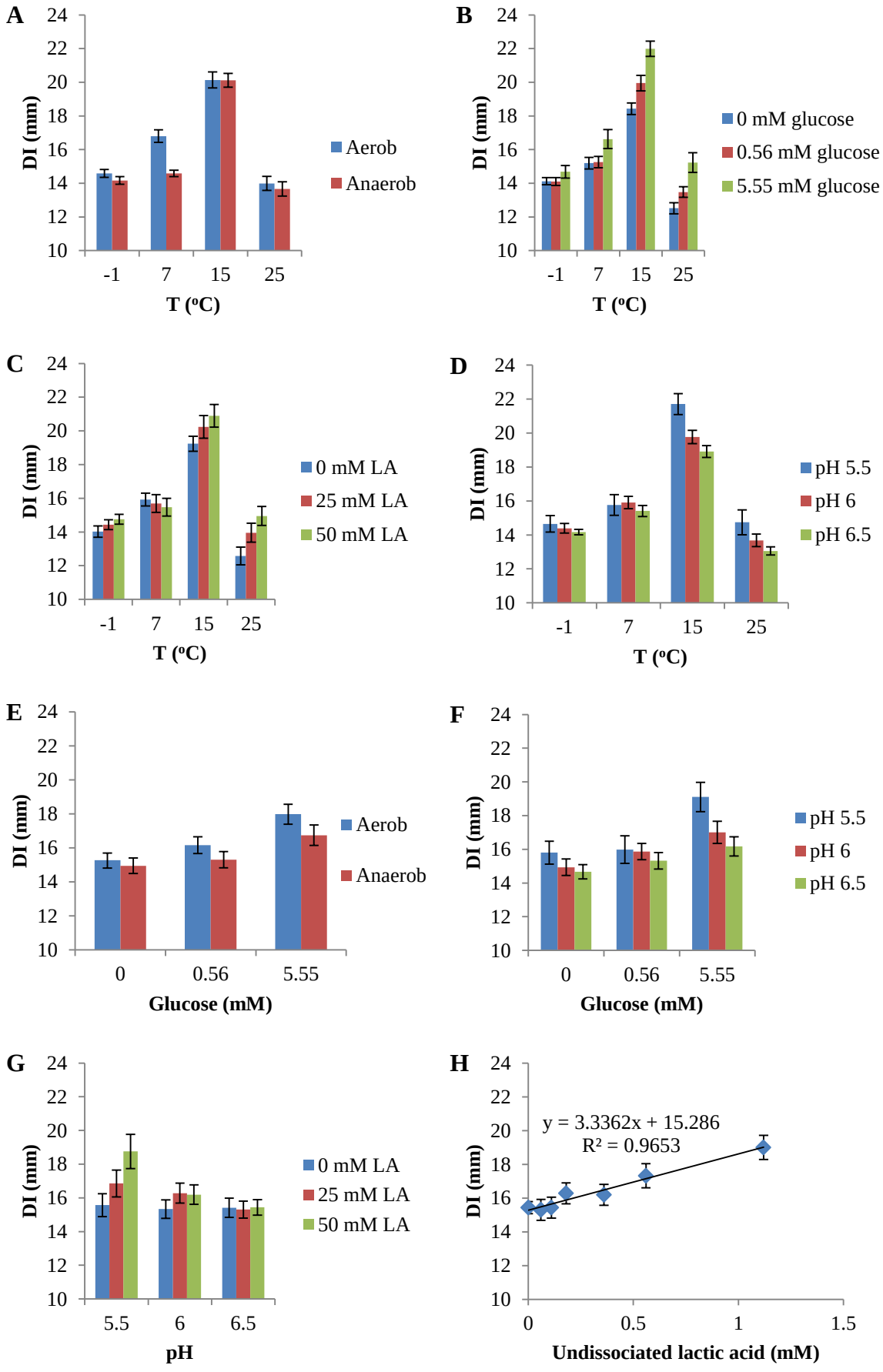

A
DI (mm)
T (ºC)
Aerob
Anaerob
B
0 mM glucose
0.56 mM glucose
5.55 mM glucose
C
0 mM LA
25 mM LA
50 mM LA
D
pH 5.5
pH 6
pH 6.5
E
Glucose (mM)
F
G
pH
H
y = 3.3362x + 15.286
R² = 0.9653
Undissociated lactic acid (mM)

**FIG 3** Interactions between environmental factors affecting diameter of inhibition zone (DI).
For panels A–G, interactions between factors were significant (F-test, $P < 0.05$). Panel H shows a linear regression between DI and undissociated lactic acid.
Error bars are standard error of the mean.

**Model**. As mentioned above, DI did not linearly correlate with temperature from 7 to 25°C, but the relationship was linear from -1 to 15°C (Fig. 2A). In addition, a significant difference in DI was observed with atmosphere (Fig. 2B), with aerobic conditions resulting in higher DI than anaerobic conditions. Therefore, DI was modelled at 25°C, and at -1 to 15°C, separately, under aerobic and anaerobic conditions, respectively. The formulae were as follows:

$$25^{o}\text{C, aerobic: } DI = 13.04 + 0.12*[\text{glucose}] - 0.629*10^{(6-pH)} + \frac{3.396*[LA]}{10^{(pH-3.86)}+1} + 0.244*[\text{glucose}]*10^{(6-pH)} \quad (R^2 = 0.724) \quad (2)$$

$$25^{o}\text{C, anaerobic: } DI = 12.139 + 0.254*[\text{glucose}] - 0.542*10^{(6-pH)} + \frac{5.107*[LA]}{10^{(pH-3.86)}+1} + 0.152*[\text{glucose}]*10^{(6-pH)} \quad (R^2 = 0.905) \quad (3)$$

$$-1^{o}\text{C}–15^{o}\text{C, aerobic: } DI = 13.61 + 0.28*T + 0.257*[\text{glucose}] - 0.198*10^{(6-pH)} + \frac{2.253*[LA]}{10^{(pH-3.86)}+1} + 0.0218*T*[\text{glucose}] \quad (R^2 = 0.786) \quad (4)$$

$$-1^{o}\text{C}–15^{o}\text{C, anaerobic: } DI = 13.55 + 0.319*T + 0.0413*[\text{glucose}] + 0.0278*T*[\text{glucose}] \quad (R^2 = 0.676) \quad (5)$$

The model for 25°C and aerobic conditions (Eq. 2) with $R^2$ of 0.724 predicted DI with the absolute residuals (i.e., absolute difference between predicted and observed DI) not larger than 2 mm for 24 conditions and absolute residuals of 2.9, 1.9, and 2.3 mm for the other three conditions (Table B1: Appendix B). The latter three conditions were mBHI containing 50 mM lactic acid and/or with pH 5.5. Equation (3) for 25°C and anaerobic condition with $R^2$ of

0.905 gave better predictions than equation (2), and its residuals were smaller than 1.5 mm for all 27 conditions (Table B2: Appendix B). Regarding the models at -1 to 15$^{o}$C, the aerobic model contained the main effects of temperature, glucose, pH, and undissociated lactic acid, whereas the anaerobic model did not require pH or undissociated lactic acid. Both models, which were based on 81 conditions each, produced relatively larger residuals than the two models at 25$^{o}$C, the largest absolute residuals being 5.8, 4.4, and 3.2 mm for aerobic conditions (Eq. 4) and 4.8, 4.5, and 4.2 mm for anaerobic conditions (Eq. 5) (Table B3 and B4: Appendix B). Similar to the 25$^{o}$C models, the worst prediction scenarios were mBHI with pH 5.5, and/or containing the largest concentration of lactic acid (50 mM). The $R^2$ of equation 4 and 5 was 0.786 and 0.676, respectively.

**Characteristics of inhibitory compounds produced by *C. maltaromaticum* D0h**. Treatment of D0h CFS with 1 mg/ml proteolytic enzymes (papain, proteinase K, and pronase E) eliminated 100% of inhibitory properties. D0h CFS inhibitory activity was not significantly reduced by pH treatment, by temperature treatment at 37 and 70$^{o}$C, or by catalase treatment (Table 3). Inhibitory activity was slightly reduced by temperature treatment at 100$^{o}$C (10%), and lipase (11%) and $\alpha$-amylase treatments (7.8%).

## DISCUSSION

Bacterial interactions have been investigated in culture media and food model systems. For example, quorum sensing compounds extracted from meat were found to promote growth of *Pseudomonas fluorescens* and *Serratia marcescens* (Nychas et al., 2009). Conversely, *Brochothrix thermosphacta* was inhibited by the presence of LAB in an agar model system (Russo et al., 2006). Youssef et al. (2014a) found that *Carnobacterium maltaromaticum*, isolated from beef cuts stored at -1.5$^{o}$C for 160 days, mostly represented a single strain; it

was inferred that the growth of other organisms would have been suppressed by bacteriocins produced by this strain. These studies add to our understanding of how microbial species and communities form in food environments; however, to date, there have been no published studies that quantify these interactions. To address this gap, we investigated the effects of pH, lactic acid, glucose and atmosphere on interactions between two *Carnobacterium maltaromaticum* beef isolates. An *in vitro* agar matrix was used for greater control of interacting factors.

Bacteriocins are frequently produced during the exponential growth phase (Baindara et al., 2013; Ghanbari et al., 2013; Quadri et al., 1994). This is consistent with production kinetics found in this study. Inhibitory effects in D0h CFS were detected in the early exponential phase, reaching maximum levels in early stationary phase (Fig. 1).

The pH of fresh beef at the beginning of storage is approximately pH 5.5; this increases to approximately pH 6.5 for aerobic packaged beef, and decreases to approximately pH 5 for beef stored under VP and modified atmosphere conditions (Argyri et al., 2015; Jones, 2004; Lavieri and Williams, 2014). A study in our lab (M. Kaur, unpublished data) showed that the pH of Australian VP beef primal samples stored at $0^{o}$C varied from pH 5.3 to 6.

Intraspecific inhibitory activity, reflected by DI measurement, was enhanced at low pH. This agrees with Ganzle et al. (1999), who reported nisin, sakacin P and curvacin A increased inhibitory activity at low pH in a broth medium. Abriouel et al. (2001) considered that $H^{+}$ affected bacteriocin activity by changing the surface charge of target bacteria, thereby causing changes in conformation/oligomerization of bacteriocin peptides. Nisin is believed to have greater activity in acidic foods, due to increased solubility and stability (Balciunas et al., 2013). To better interpret pH effects on the intraspecific inhibitory activity, final cell density was measured in mBHI agar, showing low D8c density at lower pH (Fig. B1: Appendix B).

In separate experiments, D8c growth rate in mBHI broth (25°C) increased with increased pH (Fig. B2: Appendix B). Therefore, larger inhibition zones produced at lower pH might be influenced by a higher level of inhibitory compound per cell.

Lactic acid showed an overall potentiating effect on D8c inhibition by D0h CFS. To our knowledge, no reports have investigated the effect of lactic acid on bacteriocin-like activity. Nevertheless, lactic acid has a well-known inhibitory effect on the growth of pathogenic and spoilage bacteria, and is frequently applied as a food preservative (Balannec et al., 2007; Rosengren et al., 2013; Wang et al., 2013). This effect is attributed to the undissociated form of lactic acid, which has strong inhibitory activity due to lipophilic properties, enabling it to freely diffuse through bacterial membranes (Biesta-Peters et al., 2010; Lindblad and Lindqvist, 2010; Rosengren et al., 2013; Shelef, 1994). A significant interaction was seen between lactic acid and pH on DI, with lactic acid producing a greater effect at low pH (5.5) compared to higher pH (6 and 6.5) (Fig. 3G) and showing a positive linear relationship between DI and undissociated lactic acid (Fig. 3H). Hence, we propose that the effect of lactic acid results from its undissociated form. This study also demonstrated that higher lactic acid levels in mBHI agar resulted in a lower final cell density of D8c (Fig. B1: Appendix B), indicating DI might be influenced by a higher level of inhibitory compound per target cell.

Inhibitory activity was greater in the presence of 5.5 mM compared to 0.56 mM glucose, and also when no glucose was added to mBHI agar (Fig. 2C). *Carnobacterium maltaromaticum* utilizes glucose and produces organic acids (Afzal et al., 2013; Mora et al., 2003), producing 0.21 mole of L-lactic acid per mole of glucose (Borch and Molin, 1989). To investigate the mechanism of the glucose effect on DI, pH was measured in mBHI agar incubated at 15$^{o}$C. Average pH decreased 0.4 units in 5.5 mM of glucose, which was greater than the pH decrease observed in 0 and 0.56 mM glucose, 0.09 and 0.1, respectively. Therefore, it is possible the enhancing effect of glucose on the intraspecific inhibitory activity

is due to increased lactic acid, which decreases pH during growth of D8c via glucose metabolism. This idea is consistent with interactions between glucose and pH (Fig. 3F). For 5.55 mM glucose, DI was more sensitive to pH change compared to 0 and 0.56 mM glucose. This interaction may be a reflection of the interaction between lactic acid and pH. Similarly, final cell density was lower in mBHI agar containing higher glucose levels (Fig. B1: Appendix B). Other studies have also observed that the inhibitory activity of two-component bacteriocins, such as lactocin 705 and lacticin 3147, is enhanced when target cells are energized due to the take up of glucose (Castellano et al., 2003; McAuliffe et al., 1998).

The inhibition by D0h CFS was greater under aerobic than under anaerobic conditions (Fig. 2B). According to Afzal et al. (2013), glucose metabolism by *C. maltaromaticum* LMA28 is higher in the presence of oxygen, and consequently, the production of lactic acid increases under aerobic conditions. This is likely the case in our study, where enhanced antibacterial activity of D0h CFS may result from increased production of lactic acid. It could also explain the interaction observed between atmosphere and glucose, although it was only marginally significant ($P = 0.055$) (Fig. 3E), in which the difference of DI between aerobic and anaerobic conditions was the greatest at 5.55 mM glucose.

Due to low growth rates at -1, 7, and 15$^{o}$C, mBHI agar was incubated for longer time periods (Table 2), compared to 25$^{o}$C. Importantly, preliminary tests demonstrated DI did not increase with increased incubation time (Fig. B3: Appendix B). Inhibitory activity did not linearly correlate with temperature, where DI was the greatest at 15$^{o}$C, compared to -1, 7 and 25$^{o}$C (Fig. 2A). Henry et al. (1995) reported the lethal effect of carnocin CP5 was lower, but more prolonged, in the range of 7 to 30$^{o}$C; however, Stoffels et al. (1992a) found a bacteriocin produced by *C. maltaromaticum* had no effect at 4 and 15$^{o}$C. We suggest the sensitivity, or the physiological state, of D8c target cells to D0h CFS could be affected by temperature (Jacquet et al., 2012). For instance, bacteriocins are known to interact with the

cytoplasmic membrane of sensitive bacteria (Balciunas et al., 2013; Cotter et al., 2013; Diep et al., 2007; Hechard and Sahl, 2002; Henning et al., 1986), and Jacquet et al. (2012) report that the effect of class IIa bacteriocins depends on the physiological state of target bacteria.

Combining the effects of five factors (216 conditions in total), predictive models for DI were developed for aerobic and anaerobic conditions, respectively (Eq. 2–5). The models produced relatively large residuals under certain growth conditions with the lowest pH (5.5) and/or the largest concentration of lactic acid (50 mM) (Table B1–B4: Appendix B). This may have been due to the weak *C. maltaromaticum* D8c growth (i.e. low final cell density as described above) under these conditions, resulting in larger experimental error in DI measurement. All models predicted intraspecific inhibitory activity of *C. maltaromaticum*, explaining at least 67.6% ($R^2$) of the variation in the response variable DI in the conditions investigated in this study. The interactions between food-sourced bacterial isolates have been investigated, but seldom been incorporated into mathematical models (Nychas et al., 2009; Russo et al., 2006; Zhang et al., 2015).

However, since the inhibitory activity of *C. maltaromaticum* D0h CFS at temperatures larger than 15$^{o}$C and lower than 25$^{o}$C was not investigated, the relationship between DI and temperature within this range is not clear. Hence, the models provided in this study are not capable of predicting DI for temperatures between 15 and 25$^{o}$C. Also, as this study focused on only four temperatures, and three levels each of pH, glucose, and lactic acid, the models are considered preliminary. Further studies could develop more robust models based on investigations of a larger spectrum of conditions for each factor.

CFS produced by *C. maltaromaticum* D0h was sensitive to proteolytic enzymes, including papain, proteinase K, and pronase E (Table 3). This indicates the inhibitory factor(s) in D0h CFS are proteinaceous. CFS inhibitory activity was partially reduced by lipase and α-amylase,

generally lowered by approximately 10%. Additional investigations using an ultrafiltration method showed the inhibitory factor(s) was less than 50 KDa (Fig. B4: Appendix B). In this regard, *C. maltaromaticum* has been reported to produce class I and II bacteriocins (Holck et al., 1994; Martin-Visscher et al., 2008; Quadri et al., 1994; Stoffels et al., 1992b; Tulini et al., 2014), ribosomally-synthesized peptides, which are usually heat-stable and have inhibitory activity against closely related species (Cotter et al., 2013). We consider the antibacterial compound(s) in D0h CFS to likely be a bacteriocin-like antimicrobial peptide(s).

This study focused on the effects of environmental conditions on the sensitivity of the target strain, D8c, to inhibitory factors produced by D0h. Experimental data were generated *in vitro* to more clearly define the effect of environmental factors, without the potential complicating factors of a complex meat matrix. Such bacteriological medium-based studies have been extensively used to understand how environments influence bacterial growth, resulting in predictive models (Campos et al., 2005; Mejlholm et al., 2010; Ross et al., 2003; Tienungoon et al., 2000), which are subsequently validated in food matrices. Future experiments will define the *in situ* effect of environmental factors on production of inhibitory compounds.

# Chapter 6

# Effect of environmental factors on the production of intraspecific inhibitory activity by *Carnobacterium maltaromaticum* D0h


## ABSTRACT

Intraspecific inhibition interactions have been observed among strains of *Carnobacterium maltaromaticum*, a lactic acid bacterial species that dominates microbial communities in vacuum-packaged (VP) beef. However, the environmental factors that influence such inhibition activities remain poorly understood. Using a full factorial design, the effect of pH (5.5, 6.0, and 6.5), lactic acid (0, 25, and 50 mM), glucose (0, 0.56, and 5.55 mM), and atmosphere (aerobic and anaerobic) on production of inhibitory compounds by *C. maltaromaticum* D0h in broth medium, against the growth of target isolate *C. maltaromaticum* D8c, was investigated. The production rate and level of inhibitory factors per $\log_{10}$ cfu were evaluated as a function of environment. pH had the most significant effect on production rate ($P < 0.0001$) and production level ($P = 0.03$). The highest production was observed at pH 6.5, followed by pH 6 and 5.5. A two-factor interaction was observed between glucose and pH, and lactic acid and pH. Lactic acid reduced the production rate in broth media at pH 5.5, whereas lactic acid did not have a significant effect at pH 6 or 6.5. Production rate was enhanced by glucose at pH 6.5, but was reduced at pH 5.5. Atmosphere did not significantly affect inhibitory factor production. This study extends our understanding of the effects of VP beef relevant environmental factors on intraspecific interactions between *C. maltaromaticum* strains.

## INTRODUCTION

Bacterial interactions occur in various environments, including food (Blana and Nychas, 2014; Faust and Raes, 2012; Gobbetti et al., 2007; Gram et al., 2002; Moller et al., 2013). Interactions among foodborne bacteria play an important role in influencing the composition of microbial communities, which can further affect shelf-life (Joffraud et al., 2006; Metaxopoulos et al., 2002). For example, in meat environments a decrease in meat spoilage microflora has been observed in the presence of lactic acid bacteria (LAB) (Metaxopoulos et al., 2002; Russo et al., 2006). In addition, meat extracts containing quorum sensing compounds have been shown to increase the growth rate of *Serratia marcescens* and *Pseudomonas fluorescens* (Nychas et al., 2009).

*Carnobacterium maltaromaticum* is a LAB species that often dominates vacuum-packaged (VP) meat, which can also contain species of *Enterobacteriaceae*, *Shewanella*, and *Brochothrix thermosphacta* (Doulgeraki et al., 2012; Jones, 2004; Sakala et al., 2002). Various strains of *C. maltaromaticum* produce antibacterial compounds, including bacteriocins and organic acids, which can inhibit other, as well as the strains of the same species (Holck et al., 1994; Martin-Visscher et al., 2008). Competition between *Carnobacterium* strains, including the effects of inhibitory compounds, may impact community composition within the food. The findings of Youssef et al. (2014a) indicate *C. maltaromaticum* strain G117 grew faster than other strains of *C. maltaromaticum* and *C. divergens* in VP beef primal cuts, and that bacteriocins produced by G117 may have been responsible for the observed suppressed growth of other strains.

Environmental factors can play an important role in controlling the production of antibacterial compounds by LAB (Biswas et al., 1991; Himelbloom et al., 2001; Mataragas et al., 2003). Himelbloom et al. (2001), investigating the use of *C. maltaromaticum* as

protective cultures in smoked salmon, found media composition and sodium chloride affected bacteriocin production. Glucose and pH were also found to be important factors influencing or regulating bacteriocin production by *C. maltaromaticum* (Khouiti and Simon, 2004; Schillinger et al., 1993).

In our previous studies (Chapter 5), *C. maltaromaticum* isolate D0h produced bacteriocin-like substances, which inhibited various isolates cultured from VP beef, including *C. maltaromaticum* D8c. Temperature, pH, atmosphere (aerobic and anaerobic), lactic acid, and glucose significantly affected sensitivity of D8c to D0h cell-free supernatant (CFS). However, the effect of environmental factors on production of inhibitory substances must also be understood to explain two-way interactions between *C. maltaromaticum* D0h and D8c. Therefore, the aim of this study was to investigate the *in vitro* effects of environmental factors relevant to VP beef, including pH, atmosphere, glucose and lactic acid, on production of inhibitory compounds by *C. maltaromaticum* D0h.

## MATERIALS AND METHODS

**Bacterial strains and culture preparation.** Effector isolate *Carnobacterium maltaromaticum* D0h and target strain *C. maltaromaticum* D8c were isolated from Australian VP beef, and have been described in previous studies (Small et al., 2012; Zhang et al., 2015).

The strains were separately inoculated into 1 ml of brain heart infusion broth (BHI; Amyl Media, Ltd., Australia) from a colony isolated on tryptone soy agar (TSA; Oxoid, Ltd., Australia), and incubated at 25$^{o}$C for 24 h. The cultures were then inoculated into fresh BHI, incubated at 25$^{o}$C for 24h, and used for experiments.

**Experimental design and modified BHI.** A full factorial design (2 × 3 × 3 × 3) was applied, including atmosphere (aerobic and anaerobic), lactic acid (0, 25, and 50 mM), glucose (0, 0.56, and 5.55 mM), and pH (5.5, 6, and 6.5). Fifty-four combinations of the four environmental factors were tested, in total. BHI without glucose (AM11-NG, Amyl Media, Ltd., Australia; the medium contained 17.5 g/l brain heart infusion solid, 5 g/l sodium chloride, 10 g/l blended peptone no. 1, and 2.5 g/l di-sodium hydrogen orthophosphate) was applied as the basal medium for twenty-seven formulations of modified BHI medium (mBHI), based on the factorial design (Table 1) (Chapter 5).

**TABLE 1** Lactic acid, glucose, and pH of modified BHI medium.

| Medium (#) | Lactic acid (mM) | Glucose (mM) | pH | UndisLA (mM)[a] |
|---|---|---|---|---|
| 1 | 0 | 0 | 5.5 | 0 |
| 2 | 0 | 0 | 6 | 0 |
| 3 | 0 | 0 | 6.5 | 0 |
| 4 | 25 | 0 | 5.5 | 0.56 |
| 5 | 25 | 0 | 6 | 0.18 |
| 6 | 25 | 0 | 6.5 | 0.06 |
| 7 | 50 | 0 | 5.5 | 1.12 |
| 8 | 50 | 0 | 6 | 0.36 |
| 9 | 50 | 0 | 6.5 | 0.11 |
| 10 | 0 | 0.56 | 5.5 | 0 |
| 11 | 0 | 0.56 | 6 | 0 |
| 12 | 0 | 0.56 | 6.5 | 0 |
| 13 | 25 | 0.56 | 5.5 | 0.56 |
| 14 | 25 | 0.56 | 6 | 0.18 |
| 15 | 25 | 0.56 | 6.5 | 0.06 |
| 16 | 50 | 0.56 | 5.5 | 1.12 |
| 17 | 50 | 0.56 | 6 | 0.36 |
| 18 | 50 | 0.56 | 6.5 | 0.11 |
| 19 | 0 | 5.55 | 5.5 | 0 |
| 20 | 0 | 5.55 | 6 | 0 |
| 21 | 0 | 5.55 | 6.5 | 0 |
| 22 | 25 | 5.55 | 5.5 | 0.56 |
| 23 | 25 | 5.55 | 6 | 0.18 |
| 24 | 25 | 5.55 | 6.5 | 0.06 |
| 25 | 50 | 5.55 | 5.5 | 1.12 |
| 26 | 50 | 5.55 | 6 | 0.36 |
| 27 | 50 | 5.55 | 6.5 | 0.11 |

Note: This table is cited from Chapter 5.

[a] UndisLA, undissociated lactic acid, is the calculated concentration based on the corresponding concentration of lactic acid and pH ( $[\text{UndisLA}] = [\text{Lactic acid}]/(10^{(\text{pH}-3.86)}+1)$ ).

**Inhibitory compound production**. Individual wells of a 24-well plate received 1.8 ml of mBHI, and were then inoculated with 200 µl of *C. maltaromaticum* D0h culture, for a final cell density of $10^5$ cfu/ml. Two replicate wells were used for each test condition. Plates were incubated at 25$^{o}$C, aerobically and anaerobically, respectively. Anaerobic condition (< 1.0%

$O_2$, ≥ 13% $CO_2$) was produced using a BD GasPak™ EZ Anaerobe Pouch System (BD, Australia).

In our previous study (Chapter 5), inhibitory effect of *C. maltaromaticum* D0h was detected in the exponential growth phase. Therefore, mBHI cultures were sampled with a sterile syringe at 2 h intervals when the optical density ($OD_{600}$) reached 0.05. $OD_{600}$ was measured spectrophotometrically (SPECTROstar Nano Absorbance Reader, Germany) in a 96-well plate. Cell-free supernatant (CFS) was obtained via filtering a mixture of two replicate samples through a 0.2 µm pore-sized filter (Corning®, Germany), and then serial dilutions were prepared.

The concentration of inhibitory compounds in CFS was measured by the agar overlay method described in our previous study (Chapter 5). Briefly, the agar medium (pH 6.5 ± 0.1) was made with 37 g/l BHI, 5.06 g/l L(+)-lactic acid (Scharlab, Spain), and 15 g/l agar. Ten millilitre of molten medium (50$^{o}$C) was mixed with approximate $10^5$ cfu of *C. maltaromaticum* D8c, and then poured over the previously hardened agar, made from the same medium. After air-drying in a hood, 10 µl of each CFS dilution was dropped onto the solidified agar surface. The agar plates were incubated at 25$^{o}$C for 48 h. The highest dilution of CFS showing inhibition was designated as one arbitrary inhibition unit (AU) (Ghanbari et al., 2013). Each test of the 54 conditions was performed twice.

**Cell density calculation**. To calculate the cell density in culture medium, the correlation between cell density ($log_{10}$ cfu/ml) and $OD_{600}$ was determined. Overnight (24 h) cultures of *C. maltaromaticum* D0h were diluted in two-fold serial dilutions, using BHI. For each dilution, $OD_{600}$ was measured and cell density determined by plate count. The linear regression between the two variables was calculated in Excel ® (v2010; Microsoft Corp). The cell density of each sample was then determined from a regression equation.

**Calculation of inhibitory compound production rate and level.** The relationship between sampling time (h) and concentration of inhibitory compounds (AU/ml) was calculated in Excel, and the production rate (AU/ml/h) was determined by linear regression. Linear regression was also used to associate concentration of inhibitory compounds and culture cell density. Production of inhibitory compounds by D0h per log cfu was calculated from the slope of the regression line, and designated as $\log_{10}$ AU/log cfu.

The effects of environmental factors on production rate and production level were evaluated by the F-test. If the overall effect was significant (F-test, $P < 0.05$), a student *t* test was used to identify pairwise difference. These tests were performed using the general linear modelling procedure in SAS (v9.3; SAS, Inc., Rockville, MD).

The production rate by *C. maltaromaticum* D0h under different combinations of environmental factors, including pH, glucose, and lactic acid, was plotted in 3D-scatter diagrams for aerobic and anaerobic conditions, respectively, using the Scatterplot3D package in the software R (v3.2.2 [CRAN; http://cran.r-project.org]).

## RESULTS

**Production rate.** *pH*. Among all environmental factors, pH had the most significant overall effect ($P < 0.0001$, F-test) on inhibitory compound production by *C. maltaromaticum* D0h (Fig. 1A). The production rate was highest at pH 6.5 (117.4 AU/ml/h), followed by pH 6 (95.2 AU/ml/h). At pH 5.5, inhibitory factor production decreased dramatically, showing an average of 29.8 AU/ml/h for all combinations (Fig. 1A), and was not detected in medium containing 50 mM lactic acid (medium#7, 16, and 25 in Table 1) under anaerobic conditions

(data not shown), or at pH 5.5 in medium containing 25 mM lactic acid and 5.55 mM glucose (medium #22 in Table 1), incubated aerobically and anaerobically.

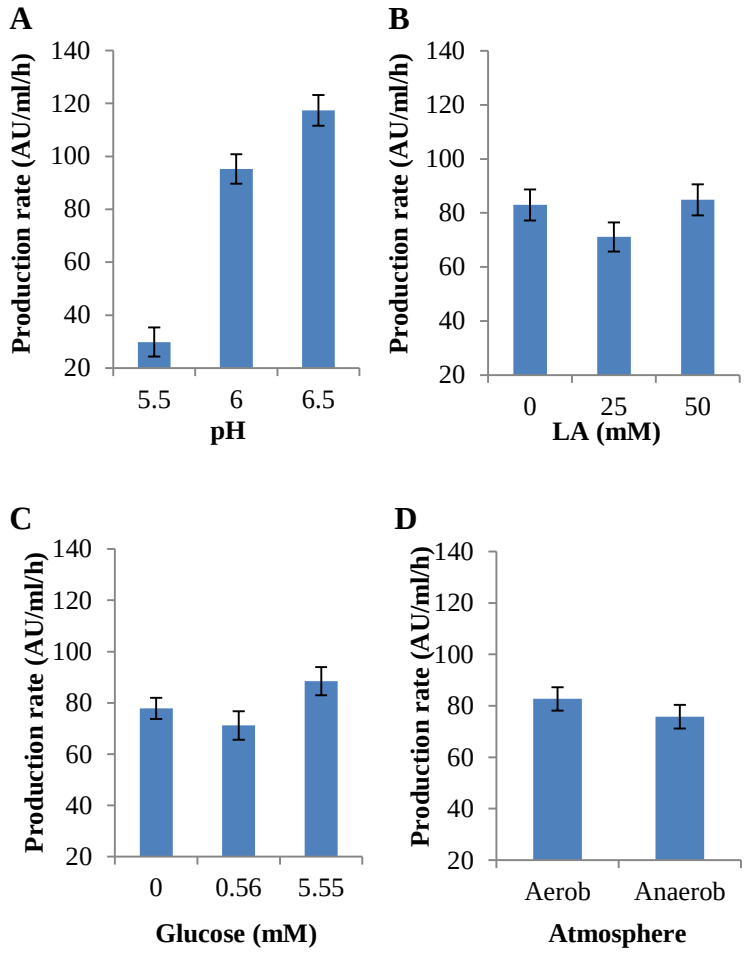



**FIG 1** Effect of environmental factors on inhibitory compound production rate.

The *P* value of F-test for pH, lactic acid (LA), glucose, and atmosphere was <0.001, 0.0392, 0.1544, and 0.2313, respectively.

Error bar represents standard error of mean.

*Lactic acid*. Interactions between lactic acid and pH were observed (*P* = 0.0043, Fig. 2A). Lactic acid had a significant effect (*P* = 0.0004) at pH 5.5, but not at pH 6 (*P* = 0.1809) and 6.5 (*P* = 0.0651). At pH 5.5, production rate was the greatest in mBHI without lactic acid

(46.6 AU/ml/h), followed by 25 and 50 mM of lactic acid (21.3 and 22.6 AU/ml/h, respectively).

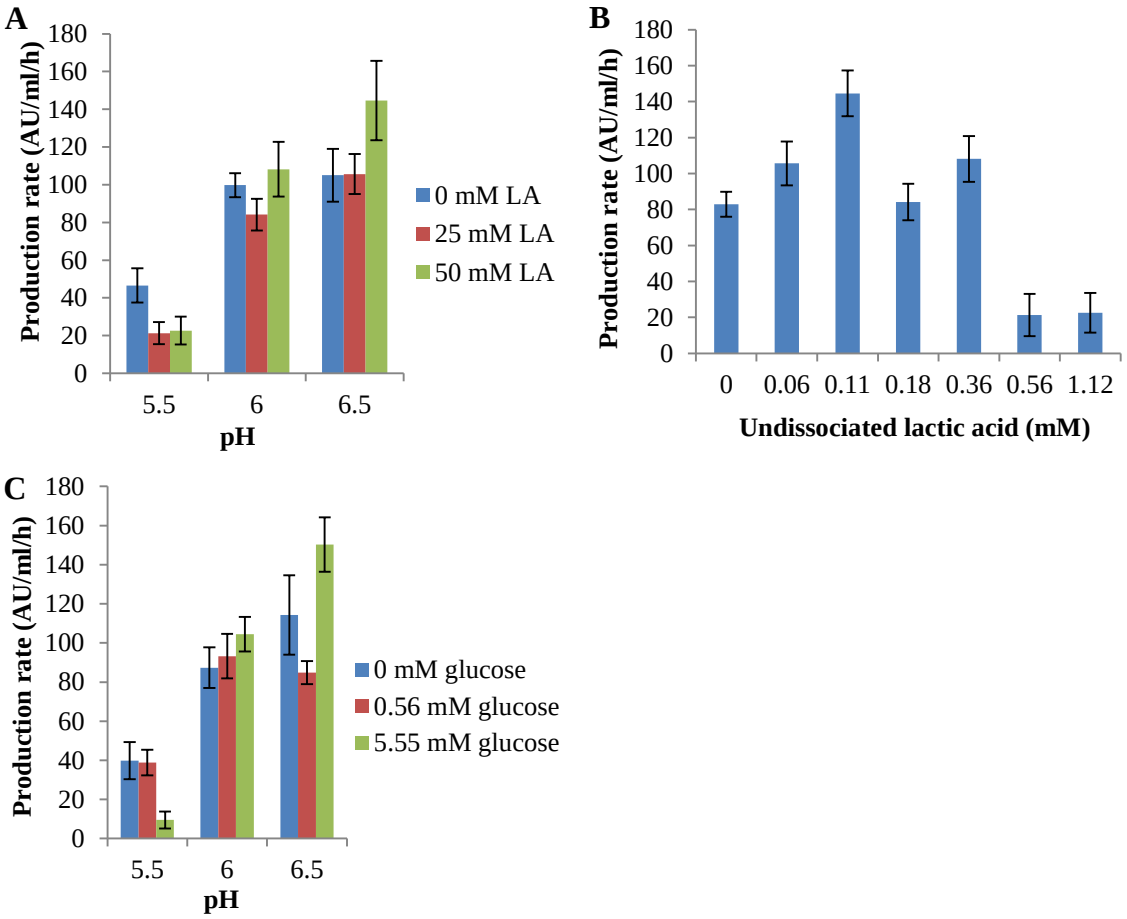



**FIG 2** Interaction between environmental factors affecting inhibitory compound production rate. *P* value of F-test in panel A, B, and C was 0.0043, < 0.0001, and 0.0002, respectively. Error bar represents standard error of mean.

Due to interactions between pH and lactic acid, the effect of undissociated lactic acid was further investigated (Fig. 2B). Interestingly, the production rate by *C. maltaromaticum* D0h did not linearly correlate with the concentration of undissociated form of lactic acid. Production rate was the highest (144.6 AU/ml/h) and lowest (21.3 and 21.6 AU/ml/h) in the media with 0.11, 0.56, and 1.12 mM undissociated lactic acid, respectively (Fig. 2B).

*Glucose*. Glucose had different effects at each of the three pH levels. Specifically, at pH 5.5 it reduced inhibitor production rate from 39.8 AU/ml/h (no glucose) to 38.8 (0.56 mM glucose), and to 9.5 (5.55 mM glucose) ($P$ = 0.0005) (Fig. 2C). However, it enhanced production rate from 114.3 (no glucose) and 84.8 (0.56 mM glucose) to 150.3 AU/ml/h (5.55 mM glucose) at pH 6.5 ($P$ = 0.01). The presence of glucose had no significant effect at pH 6 ($P$ = 0.473).

*Interactions among pH, lactic acid, and glucose.* A significant ($P$ = 0.0431) three-factor interaction was observed for pH, lactic acid, and glucose. The interaction between glucose and pH decreased with the presence of lactic acid; $P$ values were 0.0003, 0.0431, and 0.1291, for 0, 25, and 50 mM lactic acid, respectively (data not shown). Glucose also reduced the interaction between lactic acid and pH, which was significant only when there was no glucose in the medium ($P$ = 0.0240) (data not shown).

*Atmosphere*. The production rate of inhibitor was not significantly affected by atmosphere ($P$ = 0.2313); however, there was a slight difference in production rate between aerobic (82.7 AU/ml/h) and anaerobic (75.7 AU/ml/h) conditions (Fig. 1D).

**Inhibitor production level.** The equation describing cell density (log cfu/ml) and optical density (log $OD_{600}$) was (Fig. 3): Cell density = Optical density * 1.1168 + 9.4726.

The environmental factor significantly affecting production of inhibitor per cfu was pH ($P$ = 0.03), in contrast to lactic acid ($P$ = 0.6723), glucose ($P$ = 0.1357), and atmosphere ($P$ = 0.7681) (Fig. 4). Production level was lower at pH 5.5 (0.8 log AU/log cfu) compared to pH 6 (1.4 log AU/log cfu) and 6.5 (1.2 log AU/log cfu) (Fig. 4A).

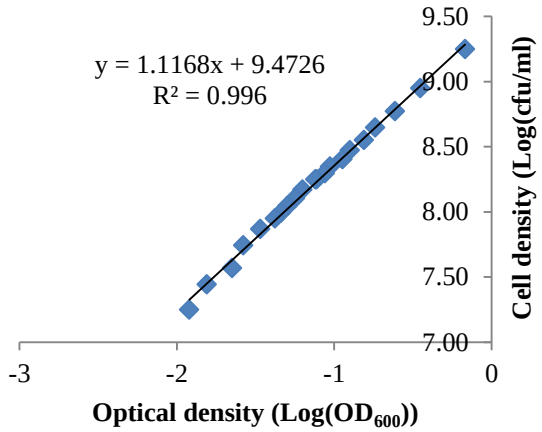



**FIG 3** Linear regression between cell density and optical density of *C. maltaromaticum* D0h in culture medium.

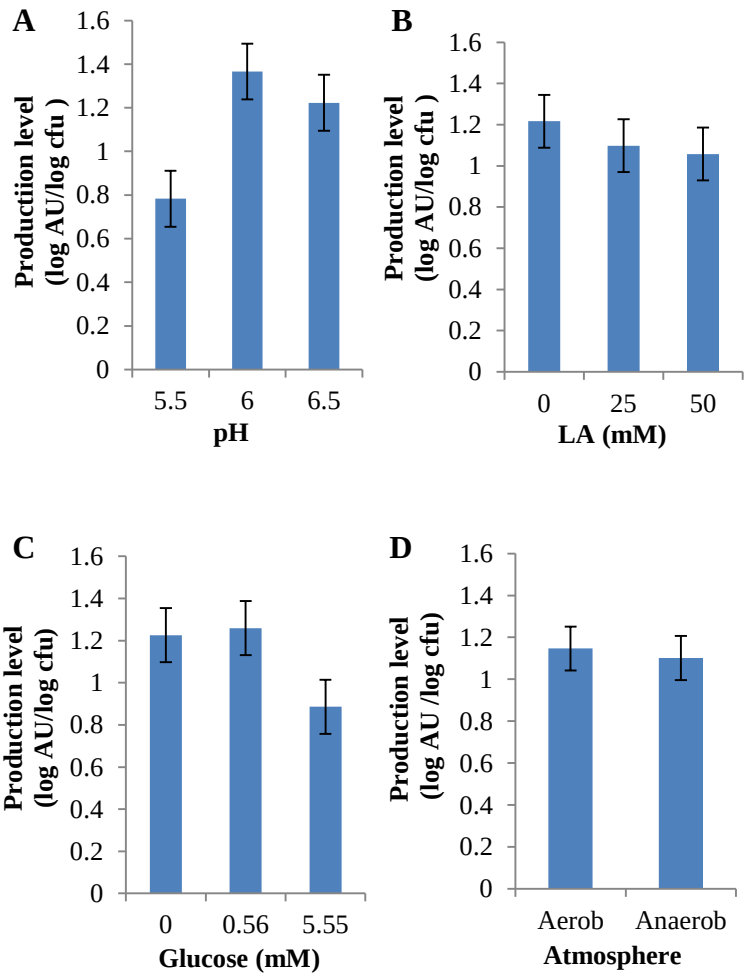



**FIG 4** Effect of environmental factors on production of inhibitory factor per cfu (log AU/log cfu). *P* value of F-test for panel A, B, C, and D is 0.03, 0.6723, 0.1357, and 0.7681, respectively.

Error bar represents standard error of mean.

## DISCUSSION

Little is known about how bacterial interactions influence development of the bacterial community in meat products, including the spoilage process (Blana and Nychas, 2014; Perez-Gutierrez et al., 2013; Wolfe et al., 2014). Our earlier study showed that environmental factors relevant to VP beef (i.e. atmosphere, pH, lactic acid, and glucose), affected sensitivity of *C. maltaromaticum* D8c to CFS produced by *C. maltaromaticum* D0h (Chapter 5). The present study extended these findings by determining how similar environmental factors influence production of inhibitors by *C. maltaromaticum* D0h.

pH was the most important environmental factor affecting inhibitory factor production by *C. maltaromaticum* D0h, reducing both production rate and level of inhibitory factor per cfu, between pH 5.5 and 6.5 (Fig. 1A and 4A). Khouiti and Simon (2004) found that *C. maltaromaticum* 213 did not produce carnocin in modified MRS medium with pH below 6.5. Also, *C. maltaromaticum* LV 61 did not produce bacteriocin at pH 5.0 (Schillinger et al., 1993). *Carnobacterium maltaromaticum* D0h produced small or no detectable levels of inhibitory factors in mBHI medium when the initial pH was 5.5 (Fig. 1A). In this regard, relatively high pH has been shown to be optimal for production of bacteriocins by *C. maltaromaticum* (Khouiti and Simon, 2004; Schillinger et al., 1993). This might result from a bacterial strategy to shift energy from biosynthesizing bacteriocins and translocating them to the external medium, to maintaining internal pH in high $H^+$ environments (Khouiti and Simon, 2004; Papagianni and Sergelidis, 2015). Other research in our laboratories (Kaur et al., unpublished data), demonstrated the pH of VP beef stored at $0^oC$ began at ~5.5, increased to approximately pH 6 at ~30 d, and then gradually decreased to pH 5.5. Production of

inhibitory compounds by *C. maltaromaticum* D0h may follow a similar pattern, which increase at the early storage and decrease thereafter.

Lactic acid displayed a significant effect on production rate of inhibitory compounds when the initial pH of mBHI was 5.5 (Fig. 2A). This may be because the concentration of undissociated lactic acid was relatively high at pH 5.5, compared to pH 6 and 6.5. Undissociated form of lactic acid is reported to have an inhibitory effect on bacterial growth (Aryani et al., 2015; Biesta-Peters et al., 2010). The lipophilic property enables it to freely diffuse across the bacterial cell membrane. Once the acid enters the bacterial cell, it dissociates with the release of protons into cytoplasm, thereby reducing internal pH (Brul and Coote, 1999; Cotter and Hill, 2003). We found inhibitory factor production rate decreased with increasing concentration of undissociated lactic acid above 0.11 mM (Fig. 2B).

Glucose is an important carbon source for LAB metabolism, including bacteriocin production (Biswas et al., 1991; Khouiti and Simon, 2004; Vignolo et al., 1995). The production rate of inhibitory factor by *C. maltaromaticum* D0h was enhanced by relatively high levels of glucose at pH 6.5 (Fig. 2C). Interestingly, glucose did not similarly affect production rate at pH 5.5, but instead reduced production rate (Fig. 2C). This may have resulted from growth inhibition induced by organic acids produced by *C. maltaromaticum* via glucose metabolism, further decreasing medium pH (Afzal et al., 2013; Mora et al., 2003).

To better define this effect, we measured the pH change of culture medium containing 0, 0.56, and 5.55 mM glucose after 48 hour incubation. pH decreased 0.49 units at 5.55 mM glucose, yet no remarkable pH effect was observed at 0 and 0.56 mM glucose (data not shown). It is possible that pH 5.5 was close to the growth/no growth boundary of *C. maltaromaticum* (Edima et al., 2008; Kim and Austin, 2008; Yang et al., 2009).

Atmosphere did not have a significant effect on inhibitory compound production, although the production rate was slightly higher under aerobic conditions (Fig. 1D). It has been reported that nisin production is associated with an oxidative metabolic pathway, and can be dramatically enhanced by high concentrations of oxygen (≥ 50%) (Cabo et al., 2001). Conversely, production of lactosin by *Lactobacillus sake* L45 was greater under anaerobic conditions (Mortvedt-Abildgaa et al., 1995). Therefore, the effect of atmosphere varies with different inhibitory compounds.

In this research, the production of inhibitory factor by D0h and the sensitivity of D8h to inhibition (Chapter 5) were studied separately. Yet, it is still possible to estimate the effects of environmental factors on the net outcome of the two-way interaction (Fig. 5 and Table 2). Glucose and lactic acid increased D8c sensitivity (Table 2 and Fig. 5) at all tested pH values, and increased D0h inhibitor production at pH 6 and 6.5 (Table 2 and Fig. 5). Therefore, D0h-D8c interaction strength would be expected to be higher in environments containing lactic acid and glucose, from pH 6 to 6.5 (Table 2). However, at pH 5.5, glucose and lactic acid decreased D0h production rate and increased D8c sensitivity; in this instance, it is more difficult to predict the additive effect of these factors on the interaction strength. Similarly, relatively high concentrations of $H^+$ increased D8c sensitivity, while decreasing inhibitor production rate by D0h (Fig. 5); it is difficult to evaluate the overall effect of $H^+$ on the interaction strength between *C. maltaromaticum* strains based on the achieved data. Under aerobic conditions, D8c sensitivity was significantly higher and inhibitor production rate was slightly higher (although not significant) than under anaerobic conditions (Table 2);therefore, D0h-D8c interaction strength is expected to be enhanced in the presence of $O_2$. It is possible that residual $O_2$, especially at the beginning of storage of VP beef, may benefit inhibitor strains of *C. maltaromaticum* to compete against sensitive strains.

**TABLE 2** Summary of the effect of environmental factors on intraspecific interactions of *C. maltaromaticum* D0h and D8c.

| Factor | | Production rate | Inhibitory activity | Interaction[a] |
|---|---|---|---|---|
| $H^+$ | | **D**[a] | **I**[a] | -[b] |
| Glucose | pH 5.5 | **D** | **I** | - |
| | pH 6 | I | **I** | I |
| | pH 6.5 | **I** | **I** | I |
| Lactic acid | pH 5.5 | **D** | **I** | - |
| | pH 6 | I | I | I |
| | pH 6.5 | I | I | I |
| Atmosphere ($O_2$) | | I | **I** | I |

Note: Effects of environmental factors on intraspecific interactions between D0h and D8c are based on the results of this study and those reported in Chapter 5.

[a] Bolded font indicates the effect was significant ($P < 0.05$);

D (decrease) or I (increase) in D0h production rate or D8c sensitivity by H+, glucose, lactic acid or atmosphere.

[b] -, The change of IS was not able to be predicted under corresponding condition.

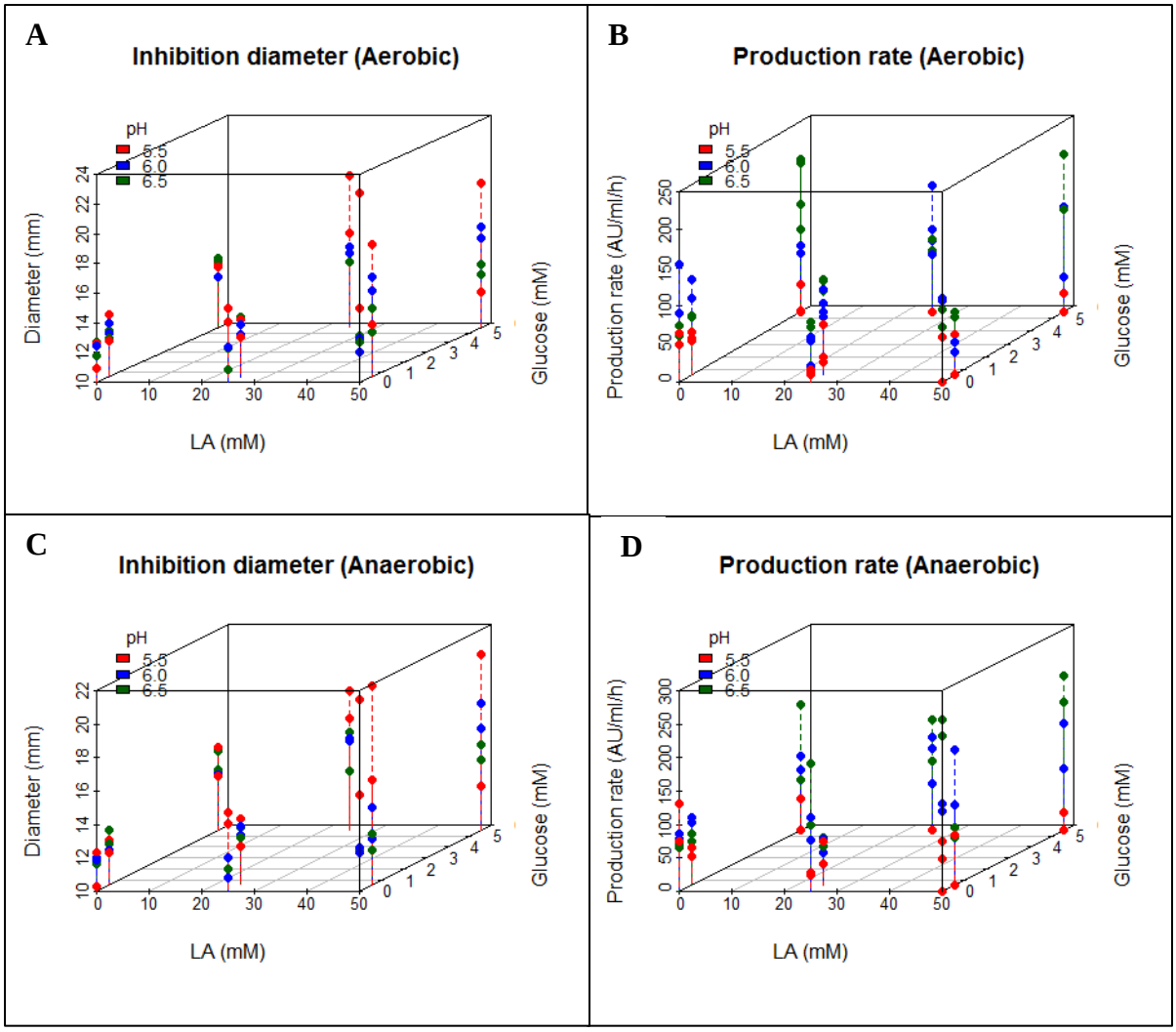



**FIG 5** *C. maltaromaticum* D8c sensitivity and D0h inhibitory production rate under different combinations of environmental factors.

A and C, sensitivity of antibacterial compounds was expressed as inhibition diameter (mm) (Chapter 5). Relatively large diameter indicates relatively larger inhibitory activity.

B and D, inhibitory compound production rate.

In conclusion, pH is one of the most important environmental factors affecting production of inhibitory factors by *C. maltaromaticum* D0h. Glucose and lactic acid affect the production rate via pH, and both factors decrease production rate at relatively low pH (5.5). Interaction between inhibiting and target strains of *C. maltaromaticum* is enhanced under aerobic conditions, and increased by glucose and lactic acid at pH 6 and 6.5.

# Chapter 7

# General discussion and conclusion

A variety of bacterial species can grow and form communities on vacuum-packaged (VP) beef. Within these communities, there is competition and cooperation occurring between species or strains (Blana and Nychas, 2014; Giaouris et al., 2015; Nychas et al., 2009; Russo et al., 2006). The long shelf-life of Australian VP beef can be partly attributed to good production control; however, it may also be due to the bacterial community composition and the interactions that occur between community members (Nychas et al., 2008; Small et al., 2012). This thesis aimed to elucidate interactions between bacteria isolated from Australian VP beef and determine the effect of environmental factors on the interactions between isolates of dominating species.

In order to study the nature of VP beef community interactions, the interactions between 39 effector and 20 target isolates were investigated at 25$^{o}$C under aerobic conditions. Both effector and target isolates represented a broad selection of bacterial taxa generally predominant on VP beef including *Carnobacterium*, *Leuconostoc*, *Brochothrix*, *Staphylococcus*, *Bacillus*, *Pseudomonas*, *Hafnia*, *Yersinia*, *Rahnella*, and *Serratia* (Brightwell et al., 2009; Doulgeraki, 2010; Doulgeraki et al., 2012; Ercolini et al., 2009; Ferrocino, 2009; Pennacchia et al., 2011).

Among these genera, LAB have been frequently reported to produce inhibitory compounds including organic acids, bacteriocins, and hydrogen peroxide (Cotter et al., 2013). This thesis research demonstrated that various isolates of LAB possessed inhibitory activity, especially *Carnobacterium maltaromaticum*, inhibiting 25-50% of 20 target isolates. Intra-

species interactions were observed for both *C. maltaromaticum* and *Carnobacterium divergens* strains. This is consistent with the published literature showing that certain strains of *Carnobacterium* are able to produce bacteriocins inhibiting closely related bacterial species, and even isolates of the same species (Martin-Visscher et al., 2008; Worobo et al., 1995). The inhibitory activity may produce a significant growth advantage to *Carnobacterium*, which is usually the dominant community component on VP red meat (Brightwell et al., 2009; Pennacchia et al., 2011; Youssef et al., 2014a).

Most *Pseudomonas* spp. isolates, except for one, inhibited almost half of the target isolates. This inhibitory activity was discerned in spot-lawn assays, in which live cells of the effector isolate were present. Similarly, the study of Aguirre-von-Wobeser et al. (2014), investigating bacteria isolated from aquatic environment and also using the spot-lawn method, found that *Pseudomonas* spp. were the most antagonistic strains. *Pseudomonas* spp. did not show a high level of inhibitory activity with cell-free supernatant (CFS) used in broth assays, indicating that either live cells are required for inhibition, or that the physiological responses of *Pseudomonas* spp. may differ when grown on solid versus broth medium.

Other inhibitory isolates included *Bacillus* spp. and *Staphylococcus epidermidis*, with the former inhibiting 80% (16 of 20) target isolates and the latter inhibiting 36.8% (7 of 19) target isolates. Strains belonging to the family *Enterobacteriaceae*, which also often dominate bacterial community of VP beef, did not exhibit high levels of inhibition.

Growth-promoting activity by foodborne bacteria has been less frequently reported, as compared to inhibition, which is probably because food microbiologists are primarily interested in studying species and conditions that reduce, not enhance, the growth of spoilage bacteria. The results from the present work found that effector isolates promoting the growth of target isolates were mostly (84%) Gram-negative bacteria, including *Pseudomonas* spp.

and *Enterobacteriaceae*; *Carnobacterium* spp. were promoted by CFS from most *Pseudomonas* spp.. Since *Pseudomonas* spp., the metabolic activities of which require oxygen (Pennacchia et al., 2011), are typically present initially on VP meat, they may potentially affect the growth of other bacterial species in communities when residual oxygen is still present. However, due to *Pseudomonas* spp. exhibiting both inhibiting (live cells) and promoting (CFS) effects as described above, the specific influence of this genus on bacterial community composition on VP beef is still not clear.

Overall, Chapter 3 of the thesis demonstrated that among a total of 774 combinations of effector and target isolates, 28.6% inhibited and 4.2 % promoted the growth of other isolates. However, the mechanisms and factors mediating these interactions are still to be elucidated; more information is needed about the effect of VP beef-related environmental factors on these interactions and the role of these interactions in shaping bacterial community structure on VP beef.

The 4th chapter broadly characterized the properties of factors responsible for isolate-isolate interactions, using eight combinations of effector and target isolates that exhibited relatively strong growth inhibition or promotion. Interactions between VP beef isolates were mediated by different mechanisms. Specifically, non-peptide compounds produced by *B. subtilis* and *Serratia* sp. were pH- and heat-stable, and promoted the growth of *Pseudomonas lundensis*. Two isolates of *C. maltaromaticum* possessing intraspecific inhibitory activity produced bacteriocin-like inhibitory compounds that were temperature and pH stable. The antibacterial compounds produced by one *B. subtilis* isolate were proteinaceous in nature, and also temperature and pH stable. In contrast, the presence of live effector cells was essential for the inhibitory activity of *Bacillus* sp., *Pseudomonas putida*, and *Pseudomonas* sp. isolates, against corresponding target isolates of *Yersinia enterocolitica*, *C. maltaromaticum*, and *B. subtilis*.; however, direct contact of live cells between target and effector isolates was not

required. Possibly, contact-dependent inhibition is not common among bacteria living on meat. This finding does not agree with either the contact-dependent inhibition via type VI secretion system widespread in Gram-negative bacteria (Boyer et al., 2009; Coulthurst, 2013; MacIntyre et al., 2010; Russell et al., 2011; Schwarz et al., 2010) or via nanotube applied by *Bacillus* sp. (Dubey and Ben-Yehuda, 2011).

As aforementioned, *C. maltaromaticum* displayed the widest inhibition spectrum compared to other LAB species. *Carnobacterium maltaromaticum* D0h strongly inhibited growth of *C. maltaromaticum* D8c (Chapter 3), and the inhibiting strain produced bacteriocin-like compounds (Chapter 4). The next two chapters of this thesis investigated the effect of environmental factors on the sensitivity of target isolate *C. maltaromaticum* D8c to CFS produced by effector isolate *C. maltaromaticum* D0h (Chapter 5), and also the effect of environmental factors on production of inhibitory factors by isolate D0h (Chapter 6).

In Chapter 5, D8c sensitivity was shown to be affected by environmental factors including pH, glucose, lactic acid, atmosphere (aerobic and anaerobic conditions), and temperature. The inhibitory activity of *C. maltaromaticum* D0h CFS was enhanced by low pH (5.5). Relatively high concentrations of $H^+$ may have affected the sensitivity of D8c to D0h CFS by a change in surface charge (Abriouel et al., 2001).

Bacteriocins inhibit closely related bacterial species by interacting with cytoplasmic membranes of target strains (Cotter et al., 2013; Diep et al., 2007; Hechard and Sahl, 2002; Henning et al., 1986). Due to decreasing pH during the storage of VP beef (Argyri et al., 2015; Jones, 2004), the inhibitory activity of *C. maltaromaticum* CFS could increase.

Lactic acid had a potentiating effect on D8c inhibition by D0h CFS; this effect may result from undissociated lactic acid, able to freely diffuse through bacterial cell membranes and

suppress bacterial growth by reducing intra-cytoplasmic pH (Biesta-Peters et al., 2010; Lindblad and Lindqvist, 2010; Rosengren et al., 2013; Shelef, 1994).

The presence of glucose enhanced inhibitory activity of D0h CFS. This may be due to *C. maltaromaticum* D8c producing more lactic acid as an end product during glucose fermentation, which also reduced the pH of its surrounding environment (Afzal et al., 2013; Borch and Molin, 1989; Mora et al., 2003). The inhibitory activity of D0h CFS on growth of D8c was higher under aerobic versus anaerobic atmosphere. This again could be associated with more rapid utilization of glucose and production of lactic acid by *C. maltaromaticum*, which has been reported more efficient in the presence of oxygen (Afzal et al., 2013).

Inhibitory activity was not linearly correlated with temperature, being the greatest at 15$^{o}$C compared to -1, 7, and 25$^{o}$C. One reason may be that varying physiological states of *C. maltaromaticum* D8c are produced when grown under different temperatures, thereby affecting the sensitivity of target bacteria to bacteriocins (Jacquet et al., 2012).

A relationship between inhibitory activity and temperature from 15–25$^{o}$C could not be defined; therefore, inhibitory activity of D0h CFS was modelled at 25$^{o}$C and at -1 to 15$^{o}$C, separately, under aerobic and anaerobic conditions, respectively. Although there were relatively large model residuals for certain growth media, such as higher lactic acid (50 mM) and/or lower pH (5.5), all four formulae explained at least 67.6% ($R^2$) of the variation for conditions investigated in this study.

In Chapter 6, pH was the most important factor dictating inhibitory factor production by *C. maltaromaticum* D0h, compared to other factors including lactic acid, glucose, and atmosphere. Relatively high concentrations of $H^+$ (pH 5.5–6.5) decreased production; D0h produced a small amount of inhibitory compound in modified brain heart infusion (mBHI) at pH 5.5. *Carnobacterium maltaromaticum* may have a strategy at relatively low pH to

maintain internal pH, rather than to expend energy to synthesize inhibitory compounds (Khouiti and Simon, 2004; Papagianni and Sergelidis, 2015). Lactic acid significantly reduced D0h production at lower pH (5.5), possibly due to the relatively high concentration of undissociated lactic acid. Production was also influenced by levels of glucose and pH, where relatively higher concentration of glucose (5.55 mM) enhanced production at pH 6.5, whereas it decreased production at pH 5.5. The presence of $O_2$ slightly increased the production of inhibitory factors although it was not significant.

Examining the findings of Chapters 5 and 6, two-way intraspecific interactions between *C. maltaromaticum* D0h and D8c can be predicted. For example, D0h-D8c interactions were greater under aerobic versus anaerobic conditions. Residual oxygen at the beginning of VP packaging could help inhibitory compound-producing strains to outcompete sensitive strains. For VP beef at pH 6 or higher, relatively high concentrations of lactic acid and glucose could provide a growth advantage to *C. maltaromaticum* inhibitor strains.

In conclusion, this research extends our understanding of interactions among bacterial strains isolated from Australian VP beef. A few LAB species, especially *Carnobacterium* spp., might be applied as protective cultures to extend VP beef shelf-life. However, the potential spoilage characteristics of candidate isolates must be investigated further, and *in situ*, before practical applications are possible.

Although complex interactions, including inhibitions and promotions exist in VP beef bacterial community, the role of these interactions in influencing dynamic community structure is not well elucidated. Future research should study the growth of mixed cultures of bacterial inhibiting, promoting, and sensitive strains. This *in vitro* study demonstrates that environmental factors including pH, temperature, glucose, lactic acid, and atmosphere affect intra-specific interactions of *C. maltaromaticum*. It is not known whether other

environmental factors influence inhibitory activity of *C. maltaromaticum*, for example, enzymes in beef and intervention chemicals such as hydrochlorous acid and peroxy-acetic acid. Future research should also investigate the influence of environmental factors on inter-species interactions, for example, interactions between LAB species and *Enterobacteriaceae* or *Brochothrix thermosphacta.*

# Appendix A

## (Chapter 3)

**TABLE A1** Interaction strength between effector and target isolates.

| Effector | | | Target | *Carnobacterium divergens* D30f | *Carnobacterium maltaromaticum* D8c | *Hafnia alvei* E30d | *Brochothrix thermosphacta* A0b | *Yersinia enterocolitica* B8b | *Yersinia* sp. D8b | *Bacillus subtilis* B30a | *Bacillus* sp. A30g | *Serratia* sp. B0h | *Serratia* sp. D0c | *Serratia* sp. D0d | *Pseudomonas lundensis* D8g | *Pseudomonas fluorescens* D8d | *Pseudomonas* sp. D0g | *Staphylococcus saprophyticus* E0c | *Staphylococcus epidermidis* F30c | *Rahnella aquatilis* B8f | *Leuconostoc carnosum* F30j | *Leuconostoc mesenteroides* B30b | *Leuconostoc* sp. F30e |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *Carnobacterium divergens* | A0a | TSA | | 0.36 | | | | | | | | | | | | | | | + | | | | |
| | | Broth | $S_{DT}$ | 0.10 | 0.18 | | | | | | | | | | | | | | | | | | |
| | | | $S_{GR}$ | | | | | | | | 0.50 | | | | | | 0.48 | | | | | | |
| | | | $S_{MPD}$ | | | | | | | | | | | | | | | | | | | | |
| | | | S | 0.03 | 0.06 | | | | | | 0.17 | | | | | | 0.16 | | | | | | |
| | A0f | TSA | | 0.31 | | | | | | | | | | | | | | | + | | | | |
| | | Broth | $S_{DT}$ | 0.14 | | | 0.15 | | | | | | | | | | 0.25 | | | | | | |
| | | | $S_{GR}$ | | | | | | | | | | | | | | | | | | | | |
| | | | $S_{MPD}$ | 0.15 | | | | | | | | | | | | 0.24 | | | | | | | |
| | | | S | 0.10 | | | 0.05 | | | | | | | | | 0.08 | 0.08 | | | | | | |

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|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | C8j | TSA | | 0.26 | | | | | | | | | | | | | | | 0.69 | | | | | | |
| | | Broth | $S_{DT}$ | | 0.15 | | 0.09 | | | | | | | | 0.17 | 0.16 | 0.29 | 0.11 | | 0.09 | | | | | |
| | | | $S_{GR}$ | | | | | | | | | | | | | 0.31 | | | | | | | | | |
| | | | $S_{MPD}$ | | | | | | | | | | | | | | | | | | | | | | |
| | | | S | | 0.05 | | 0.03 | | | | | | | | 0.06 | 0.16 | 0.10 | 0.04 | | 0.03 | | | | | |
| | D30a | Agar | | + | | | | | | | | | | | | | | | | | | | | | |
| | | Broth | $S_{DT}$ | | 0.11 | | | | 0.16 | | | | | | | | | | | | | | | | |
| | | | $S_{GR}$ | 0.61 | | | | | | | | | | | | | | | | | | | | | |
| | | | $S_{MPD}$ | 0.17 | | | | | | | | | | | | | | | | | | | | | |
| | | | S | 0.26 | 0.04 | | | | 0.05 | | | | | | | | | | | | | | | | |
| | E0j | TSA | | 0.25 | | | | | | | | | | | | | | | + | | | | | | |
| | | Broth | $S_{DT}$ | 0.14 | | | 0.18 | | | | | | | | | | | | 0.11 | 0.10 | | 0.09 | | | |
| | | | $S_{GR}$ | | | | | | | | | | | | | | | | | | | | | | |
| | | | $S_{MPD}$ | | | | | | | | | | | | | | | | | | | | | | |
| | | | S | 0.05 | | | 0.06 | | | | | | | | | | | | 0.04 | 0.03 | | 0.03 | | | |
| | F8f | TSA | | 0.28 | | | | | | | | | | | | | | | | + | | | | | |
| | | Broth | $S_{DT}$ | | | | 0.18 | | | | | | | | | 0.14 | | | | | | | | | |
| | | | $S_{GR}$ | | | | | | | | | | | | | 0.27 | | | | | | | | | |
| | | | $S_{MPD}$ | | | | | | | | | | | | | | | | | | | | | | |
| | | | S | | | | 0.06 | | | | | | | | | 0.14 | | | | | | | | | |
| *Carnobacterium maltaromaticum* | B0f | TSA | | V+ | 4.48 | + | | | | | | | | | | | | | | | | | | | |
| | | Broth | $S_{DT}$ | NG | NG | | | | 0.14 | | | | | | | 0.14 | | | 0.09 | | | | | | |
| | | | $S_{GR}$ | | | | | | 0.24 | | | | | | | 0.28 | | 0.37 | 0.36 | 0.24 | | | | | |
| | | | $S_{MPD}$ | | | | | | | | | | | | | | | | | | | | | | |
| | | | S | | | | | | 0.13 | | | | | | | 0.14 | | 0.12 | 0.15 | 0.08 | | | | | |
| | C0a | TSA | | | | | | | | | | | | | | | | | | | | | | | |
| | | Broth | $S_{DT}$ | 0.12 | 0.16 | | | | 0.11 | | | | | | | | | | | | | | | | |
| | | | $S_{GR}$ | | 0.08 | | | | | 0.22 | | | | | | | | | 0.18 | | | | | | |
| | | | $S_{MPD}$ | 0.14 | | | | | | 0.36 | | | | | | | | | | | | | | | |
| | | | S | 0.09 | 0.08 | | | | 0.04 | 0.19 | | | | | | | | | 0.06 | | | | | | |
| | C8h | TSA | | | | | | | | | | | | | | | | | | | | | | | |
| | | Broth | $S_{DT}$ | 0.11 | 0.28 | | | | 0.14 | | 0.20 | | 0.15 | 0.35 | | | 0.25 | 0.27 | | | | | | | |
| | | | $S_{GR}$ | | | | | | | | | | | | | | | | 0.46 | | | | | | |

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| | | | $S_{MPD}$ | | | | | | | | | | | | 0.24 | | | | | | | | | | | |
| | | | S | 0.04 | 0.09 | | | | 0.05 | | 0.07 | | 0.05 | 0.20 | | 0.08 | 0.09 | 0.15 | | | | | | | |
| | | TSA | | V1.09 | 4.81 | | | | | | | | | | | | | | | | | | | | | |
| | | Broth | $S_{DT}$ | NG | NG | | 0.10 | | 0.19 | | 0.19 | | 0.18 | NG | | 0.34 | NG | | | | | | | | | |
| | | | $S_{GR}$ | | | | | | 0.38 | | | | | | | | | | | | | | | | | |
| | | | $S_{MPD}$ | | | | | | | | | | | | | | | | | | | | | | | |
| | C30h | | S | | | | 0.03 | | 0.19 | | 0.06 | | 0.06 | | | 0.11 | | | | | | | | | | |
| | | TSA | | V+ | 4.82 | | | | | | | | | | | | | | | | | | 0.16 | | 0.49 | |
| | | Broth | $S_{DT}$ | NG | NG | | | | 0.27 | | 0.12 | | 0.17 | NG | | NG | NG | | | | | | 0.55 | | | |
| | | | $S_{GR}$ | | | | | | | | | | | | | | | | | | | | | | | |
| | | | $S_{MPD}$ | | | | | | | | | | | | | | | | | | | | 0.40 | | | |
| | D0h | | S | | | | | | 0.09 | | 0.04 | | 0.06 | | | | | | | | | | 0.32 | | | |
| *Carnobacterium* sp. | | TSA | | | | | | | | | | | | | | | | | | | | | | | | |
| | | Broth | $S_{DT}$ | 0.11 | 0.32 | | | | 0.20 | | 0.17 | | | 0.43 | | 0.32 | 0.16 | | | | | | | | | |
| | | | $S_{GR}$ | | | | | | | | 0.74 | | | | | | | | | | | | | | | |
| | | | $S_{MPD}$ | | | | | | | | | | | | | | | | | | | | | | | |
| | F8g | | S | 0.04 | 0.11 | | | | 0.07 | | 0.30 | | | 0.14 | | 0.11 | 0.05 | | | | | | | | | |
| *Leuconostoc carnosum* | | TSA | | | | | | | | | | | | | | | | | | | | | | | | |
| | | Broth | $S_{DT}$ | | | | | | | | | | | 0.11 | | | | | | | | | 0.16 | | | |
| | | | $S_{GR}$ | | | | | | | | | | | | | | | | | | | | 0.32 | | | |
| | | | $S_{MPD}$ | | | | | | | | | | | | | | | | | | | | 0.30 | | | |
| | F30d | | S | | | | | | | | | | | 0.04 | | | | | | | | | 0.26 | | | |
| | | TSA | | | | | | | | | | | | | | | | | | | | | | | | |
| | | Broth | $S_{DT}$ | | | | | | | | | | | | | | 0.14 | | | | | | | | | |
| | | | $S_{GR}$ | | | | | | | | | | | | | | 0.21 | | | | | | 0.29 | | | |
| | | | $S_{MPD}$ | | | | | | | | | | | | | | | | | | | | 0.20 | | | |
| | F30h | | S | | | | | | | | | | | | | | 0.12 | | | | | | 0.16 | | | |
| *Leuconostoc mesenteroides* | | TSA | | | | | | | | | | | | | | | | | | | | | | | | |
| | | Broth | $S_{DT}$ | 0.08 | 0.16 | | | | | | | | | | | | | | | | | | | | | |
| | | | $S_{GR}$ | | | | | | | | | | | | | | | | | | | | 0.05 | | | |
| | | | $S_{MPD}$ | | | | | | | | | | | | 0.18 | | | | | | | | 0.15 | | | |
| | B30b | | S | 0.03 | 0.05 | | | | | | | | | | 0.06 | | | | | | | | 0.07 | | | |
| *Brochothrix* | A8f | TSA | | | | | | | | | | | | | | | | | | | | | | | | |

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|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *thermosphacta* | | Broth | $S_{DT}$ | | 0.17 | | | | | | | | | 0.11 | | 0.13 | 0.19 | | | | | | | |
| | | | $S_{GR}$ | | 0.21 | | | | | | | | | | | | | 0.46 | | | | | | |
| | | | $S_{MPD}$ | | | | | | | | | | | | | | | | | | | | | |
| | | | S | | 0.13 | | | | | | | | | 0.04 | | 0.04 | 0.06 | 0.15 | | | | | | |
| *Staphylococcus epidermidis* | F30c | TSA | | 2.31 | 1.89 | | 0.38 | | | | | | | | | | | 0.35 | | | 0.14 | 0.96 | 1.41 | |
| | | Broth | $S_{DT}$ | 0.63 | 0.27 | | 0.05 | | | | | | | | | | | 0.46 | | | | | | |
| | | | $S_{GR}$ | 0.21 | | | | | | | | | | | | | | | | | | | | |
| | | | $S_{MPD}$ | | | | | | | | | | | | | | | | | | | | | |
| | | | S | 0.28 | 0.09 | | 0.02 | | | | | | | | | | | 0.15 | | | | | | |
| *Bacillus subtilis* | E0g | TSA | | V+ | 0.64 | V+ | 1.49 | V+ | 0.21 | | 1.62 | | | | | | | 1.40 | 1.53 | | 0.93 | 0.13 | 2.85 | |
| | | Broth | $S_{DT}$ | NG | 1.35 | | NG | | | | NG | | | NG | | 1.66 | 0.73 | NG | NG | | NG | NG | | |
| | | | $S_{GR}$ | | 0.64 | | | | | | | | | | | 0.57 | | | | | | | | |
| | | | $S_{MPD}$ | | 0.40 | | | | | 0.35 | | | | | 0.45 | 0.41 | | | | | | | | |
| | | | S | | 0.80 | | | | | 0.12 | | | | | 0.15 | 0.88 | 0.24 | | | | | | | |
| *Bacillus* sp. | A30g | TSA | | | V+ | V1 | 1.02 | 2.65 | 1.52 | + | | | + | | V+ | + | 0.25 | V+ | V+ | 1.75 | | V+ | | |
| | | Broth | $S_{DT}$ | | 0.04 | | | | | 0.33 | | | | | | 0.10 | | | | | | | | |
| | | | $S_{GR}$ | 0.20 | | | | | | | | | | | 0.36 | 0.33 | 1.09 | | | | 0.74 | | | |
| | | | $S_{MPD}$ | | | | | | | | | | | | 0.59 | | 0.36 | | | | | 0.48 | | |
| | | | S | 0.07 | 0.01 | | | | | 0.11 | | | | | 0.32 | 0.14 | 0.48 | | | | 0.25 | 0.16 | | |
| *Pseudomonas fluorescens* | B0i | TSA | | | | | V+ | | | + | | | | | | | | | V+ | | | | | |
| | | Broth | $S_{DT}$ | 0.08 | 0.13 | | | | | | | | | | | 0.14 | 0.91 | | | | | | | |
| | | | $S_{GR}$ | | | | | | | | | | | | | | | 0.22 | 0.11 | | | | | |
| | | | $S_{MPD}$ | | | | | | | | | | | | | | | | | | | | | |
| | | | S | 0.03 | 0.04 | | | | | | | | | | | 0.05 | 0.30 | 0.07 | 0.04 | | | | | |
| | C0c | TSA | | | | | | V+ | | 0.22 | + | | | | 0.30 | 0.22 | 0.37 | V+ | V0.29 | | | | | |
| | | Broth | $S_{DT}$ | | 0.12 | | | | | | | | | | 0.17 | | | | | | | | | |
| | | | $S_{GR}$ | | | | | | | | | | | | | 0.22 | | | | | | 0.32 | | |
| | | | $S_{MPD}$ | | | | | | | | | | | | | | | | | | | | | |
| | | | S | | 0.04 | | | | | | | | | | 0.06 | 0.07 | | | | | | 0.11 | | |
| *Pseudomonas fragi* | F0b | TSA | | V+ | V+ | 0.25 | V+ | V+ | | 1.68 | + | | | | | | | V0.97 | V+ | V+ | V+ | V+ | | |
| | | Broth | $S_{DT}$ | 0.08 | | | | | | | | | | | | | | | 0.07 | | | | | |
| | | | $S_{GR}$ | | 0.13 | | | | | | | | | | | | 0.45 | | | | | | | |
| | | | $S_{MPD}$ | | | | | | | | | | | | | | | | | | | | | |
| | | | S | 0.03 | 0.04 | | | | | | | | | | | | 0.15 | | 0.02 | | | | | |

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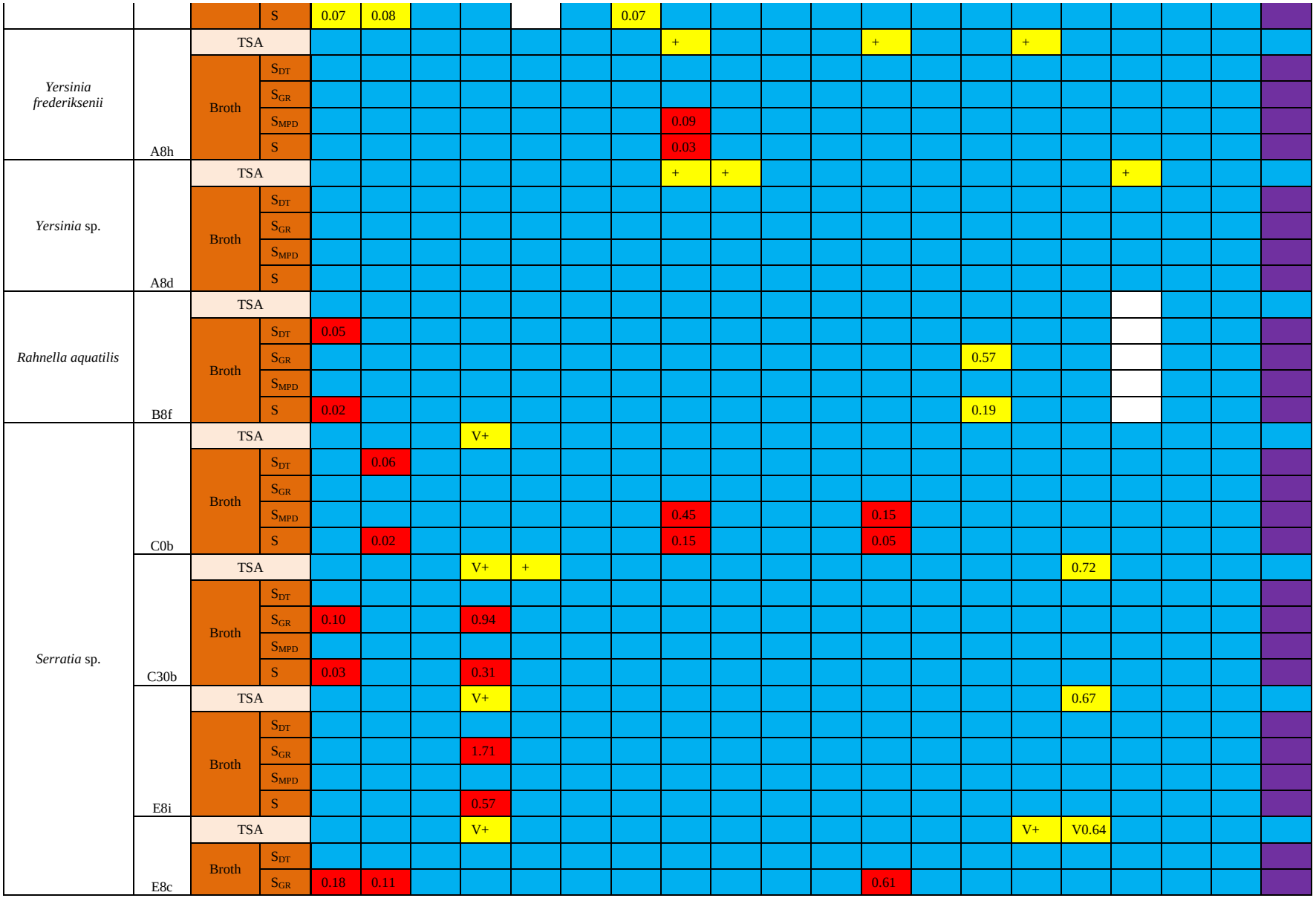
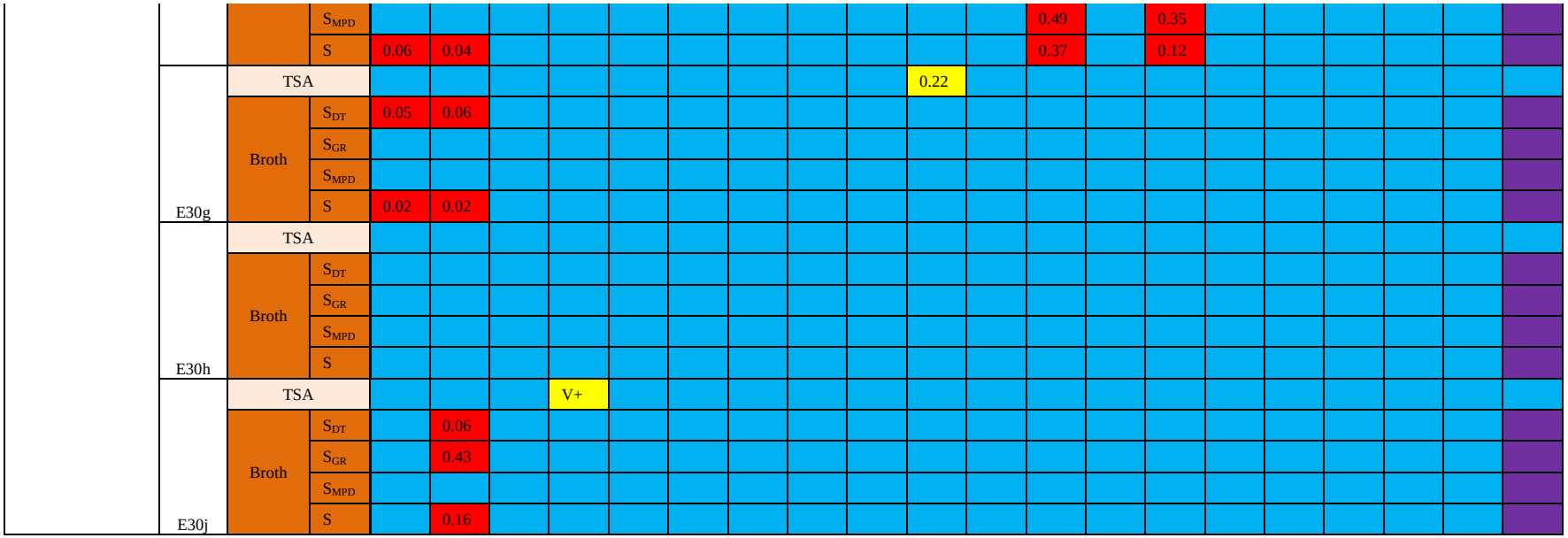
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *Pseudomonas putida* | D0b | TSA | | V4.53 | 6.04 | V2.43 | V+ | V3.33 | V2.36 | 2.67 | 3.26 | V1.49 | V0.94 | V+ | 1.42 | V0.25 | 2.30 | V2.19 | V1.52 | V1.77 | V+ | | | |
| | | Broth | $S_{DT}$ | 0.07 | 0.13 | | | | | | | | | | | | | | | | | | | |
| | | | $S_{GR}$ | | | | | | | | | | | | | | | | 0.15 | | | | | |
| | | | $S_{MPD}$ | | | | | | | | | | | | | | | | | | | | | |
| | | | S | 0.02 | 0.04 | | | | | | | | | | | | | | 0.05 | | | | | |
| *Pseudomonas* sp. | D0g | TSA | | | V+ | V+ | V+ | V+ | | 2.30 | + | | | | | | | V1.31 | V1.90 | | V+ | V+ | | |
| | | Broth | $S_{DT}$ | | 0.15 | | | | | | | | | | | | | | 0.16 | | | | | |
| | | | $S_{GR}$ | | | | | | | | | | | | | | | | | | | | | |
| | | | $S_{MPD}$ | | | | | | | | | | | | | | | | | | | | | |
| | | | S | | 0.05 | | | | | | | | | | | | | | 0.05 | | | | | |
| | E0f | TSA | | V+ | V1.78 | + | | V+ | | 0.48 | 0.27 | | | | | + | V+ | | 1.31 | 0.13 | V+ | | | |
| | | Broth | $S_{DT}$ | 0.11 | 0.15 | | | | | | | | | | 0.21 | | | | | | | | | |
| | | | $S_{GR}$ | | | | | | | | | | | | | 0.16 | | | | | | | | |
| | | | $S_{MPD}$ | | | | | | | | | | | | | | | | | | | | | |
| | | | S | 0.04 | 0.05 | | | | | | | | | | 0.07 | 0.05 | | | | | | | | |
| *Hafnia alvei* | A8e | TSA | | | | | | | | | | | | | V+ | | | | | | | | | |
| | | Broth | $S_{DT}$ | | | | | | | | | | | | | | | | | | | | | |
| | | | $S_{GR}$ | | | | | | | | | | | | | | | | | | | | | |
| | | | $S_{MPD}$ | | | | | | | | | | | | | | | | | | | | | |
| | | | S | | | | | | | | | | | | | | | | | | | | | |
| | D0f | TSA | | | | | | | | | + | | | | | | | | | | | | | |
| | | Broth | $S_{DT}$ | 0.07 | | | | | | | | | | | | | | | | | | | | |
| | | | $S_{GR}$ | | | | | | | | | | | | | | | | | | | | | |
| | | | $S_{MPD}$ | | | | | | | | | | | | | | | | | | | | | |
| | | | S | 0.02 | | | | | | | | | | | | | | | | | | | | |
| | E30e | TSA | | | | | | | | | | | | | | | | | | | | | | |
| | | Broth | $S_{DT}$ | 0.13 | | | | | | | | | | | | | | | | | | | | |
| | | | $S_{GR}$ | | | | | | | | | | | | | | | | | | | | | |
| | | | $S_{MPD}$ | | | | | | | | | | | | | | | | | | | | | |
| | | | S | 0.04 | | | | | | | | | | | | | | | | | | | | |
| *Yersinia enterocolitica* | B8b | TSA | | | | | | | | | | | + | | | | | | | | | | | |
| | | Broth | $S_{DT}$ | 0.08 | 0.15 | | | | | | | | | | | | | | | | | | | |
| | | | $S_{GR}$ | | | | | | | | | | | | | | | | | | | | | |
| | | | $S_{MPD}$ | 0.13 | 0.08 | | | | | 0.21 | | | | | | | | | | | | | | |


S 0.07 0.08 0.07
TSA + + + +
Broth $S_{DT}$
Broth $S_{GR}$
Broth $S_{MPD}$ 0.09
Broth S 0.03
Yersinia frederiksenii
A8h
TSA + + +
Yersinia sp.
A8d
TSA
Broth $S_{DT}$ 0.05
Broth $S_{GR}$ 0.57
Broth $S_{MPD}$
Broth S 0.02 0.19
Rahnella aquatilis
B8f
TSA V+
Broth $S_{DT}$ 0.06
Broth $S_{GR}$
Broth $S_{MPD}$ 0.45 0.15
Broth S 0.02 0.15 0.05
C0b
TSA V+ + 0.72
Broth $S_{DT}$
Broth $S_{GR}$ 0.10 0.94
Broth $S_{MPD}$
Broth S 0.03 0.31
C30b
TSA V+ 0.67
Broth $S_{DT}$
Broth $S_{GR}$ 1.71
Broth $S_{MPD}$
Broth S 0.57
E8i
TSA V+ V+ V0.64
Broth $S_{DT}$
Broth $S_{GR}$ 0.18 0.11 0.61
E8c
Serratia sp.




**Note:**

| | |
|---|---|
| | Inhibition |
| | Promotion |
| | No interaction |
| | The effector and target isolate is the same strain |
| | Not measured |
| $S_{DT}$ | Interaction strength calculated by comparing detection time (DT) of treatment and control |
| $S_{GR}$ | Interaction strength calculated by comparing growth rate (GR) of treatment and control |
| $S_{MPD}$ | Interaction strength calculated by comparing maximum population density (MPD) of treatment and control |
| S | Average of $S_{DT}$, $S_{GR}$ and $S_{MPD}$ |
| V | The inhibition zone showed a vague edge on TSA |

| | |
|---|---|
| NG | The target isolate did not grow |

1. Regarding the interaction in spot-lawn assay (TSA), interaction strength (S) is shown as the diameter (mm) of inhibition zone. If an inhibition zone was observed on TSA while it was too small to measure, S was regarded as level '+'.
2. For the combination which did not show target isolate growth, S is displayed as NG (no growth).

# Appendix B

## (Chapter 5)

**TABLE B1** Model prediction residuals for 25$^{o}$C and aerobic conditions.

| Medium (#) | Glucose (mM) | pH | LA (mM) | Diameter of inhibition zone (mm) | | |
|---|---|---|---|---|---|---|
| | | | | Observation | Prediction | Residual |
| 1 | 0 | 5.5 | 0 | 10.91 | 11.0508 | -0.1408 |
| 2 | 0 | 6 | 0 | 12.44 | 12.4107 | 0.02927 |
| 3 | 0 | 6.5 | 0 | 11.77 | 12.8408 | -1.0708 |
| 4 | 0 | 5.5 | 25 | 14.03 | 12.9519 | 1.07812 |
| 5 | 0 | 6 | 25 | 12.38 | 13.0213 | -0.6413 |
| 6 | 0 | 6.5 | 25 | 12.27 | 13.0348 | -0.7648 |
| 7 | 0 | 5.5 | 50 | 15.02 | 14.853 | 0.167 |
| 8 | 0 | 6 | 50 | 12.06 | 13.6318 | -1.5718 |
| 9 | 0 | 6.5 | 50 | 12.67 | 13.2288 | -0.5588 |
| 10 | 0.56 | 5.5 | 0 | 12.45 | 11.5504 | 0.89965 |
| 11 | 0.56 | 6 | 0 | 13.63 | 12.6147 | 1.01527 |
| 12 | 0.56 | 6.5 | 0 | 12.62 | 12.9513 | -0.3313 |
| 13 | 0.56 | 5.5 | 25 | 12.71 | 13.4515 | -0.7415 |
| 14 | 0.56 | 6 | 25 | 13.54 | 13.2253 | 0.31473 |
| 15 | 0.56 | 6.5 | 25 | 13.53 | 13.1453 | 0.38466 |
| 16 | 0.56 | 5.5 | 50 | 13.53 | 15.3526 | -1.8226 |
| 17 | 0.56 | 6 | 50 | 16.75 | 13.8358 | 2.91419 |
| 18 | 0.56 | 6.5 | 50 | 14.57 | 13.3394 | 1.23063 |
| 19 | 5.55 | 5.5 | 0 | 14.08 | 16.0021 | -1.9221 |
| 20 | 5.55 | 6 | 0 | 13.43 | 14.4326 | -1.0026 |
| 21 | 5.55 | 6.5 | 0 | 14.5 | 13.9362 | 0.56376 |
| 22 | 5.55 | 5.5 | 25 | 20.19 | 17.9032 | 2.28676 |
| 23 | 5.55 | 6 | 25 | 14.96 | 15.0431 | -0.0831 |
| 24 | 5.55 | 6.5 | 25 | 14.39 | 14.1303 | 0.25973 |
| 25 | 5.55 | 5.5 | 50 | 19.68 | 19.8044 | -0.1244 |
| 26 | 5.55 | 6 | 50 | 16.01 | 15.6536 | 0.35635 |
| 27 | 5.55 | 6.5 | 50 | 13.6 | 14.3243 | -0.7243 |

**TABLE B2** Model prediction residuals for 25$^{o}$C and anaerobic conditions.

| Medium (#) | Glucose (mM) | pH | LA (mM) | Diameter of inhibition zone (mm) | | |
|---|---|---|---|---|---|---|
| | | | | Observation | Prediction | Residual |
| 1 | 0 | 5.5 | 0 | 10.34 | 10.4248 | -0.0848 |
| 2 | 0 | 6 | 0 | 11.88 | 11.5971 | 0.28294 |
| 3 | 0 | 6.5 | 0 | 11.75 | 11.9678 | -0.2178 |
| 4 | 0 | 5.5 | 25 | 14.03 | 13.284 | 0.74604 |
| 5 | 0 | 6 | 25 | 12.01 | 12.5153 | -0.5053 |
| 6 | 0 | 6.5 | 25 | 11.33 | 12.2596 | -0.9296 |
| 7 | 0 | 5.5 | 50 | 15.81 | 16.1431 | -0.3331 |
| 8 | 0 | 6 | 50 | 12.3 | 13.4335 | -1.1335 |
| 9 | 0 | 6.5 | 50 | 12.35 | 12.5514 | -0.2014 |
| 10 | 0.56 | 5.5 | 0 | 11.99 | 10.8355 | 1.15455 |
| 11 | 0.56 | 6 | 0 | 12.1 | 11.8242 | 0.27581 |
| 12 | 0.56 | 6.5 | 0 | 12.48 | 12.1369 | 0.34315 |
| 13 | 0.56 | 5.5 | 25 | 12.31 | 13.6946 | -1.3846 |
| 14 | 0.56 | 6 | 25 | 13.5 | 12.7424 | 0.75761 |
| 15 | 0.56 | 6.5 | 25 | 12.83 | 12.4286 | 0.40135 |
| 16 | 0.56 | 5.5 | 50 | 16.3 | 16.5538 | -0.2538 |
| 17 | 0.56 | 6 | 50 | 14.63 | 13.6606 | 0.9694 |
| 18 | 0.56 | 6.5 | 50 | 13.1 | 12.7204 | 0.37955 |
| 19 | 5.55 | 5.5 | 0 | 13.19 | 14.4947 | -1.3047 |
| 20 | 5.55 | 6 | 0 | 13.26 | 13.8481 | -0.5881 |
| 21 | 5.55 | 6.5 | 0 | 13.56 | 13.6436 | -0.0836 |
| 22 | 5.55 | 5.5 | 25 | 18.32 | 17.3539 | 0.9661 |
| 23 | 5.55 | 6 | 25 | 15.31 | 14.7663 | 0.54372 |
| 24 | 5.55 | 6.5 | 25 | 13.52 | 13.9354 | -0.4154 |
| 25 | 5.55 | 5.5 | 50 | 20.47 | 20.2131 | 0.25694 |
| 26 | 5.55 | 6 | 50 | 16.07 | 15.6845 | 0.38551 |
| 27 | 5.55 | 6.5 | 50 | 14.2 | 14.2272 | -0.0272 |

**TABLE B3** Model prediction residuals for -1–15$^{o}$C and anaerobic conditions.

| Medium (#) | T ($^{o}$C) | Glucose (mM) | pH | LA (mM) | Diameter of inhibition zone (mm) | | |
|---|---|---|---|---|---|---|---|
| | | | | | Observation | Prediction | Residual |
| 1 | -1 | 0 | 5.5 | 0 | 13.93 | 13.9535 | -0.0235 |
| 2 | -1 | 0 | 6 | 0 | 13.27 | 13.5255 | -0.2555 |
| 3 | -1 | 0 | 6.5 | 0 | 13.86 | 13.3902 | 0.46983 |
| 4 | -1 | 0 | 5.5 | 25 | . | 15.2151 | . |
| 5 | -1 | 0 | 6 | 25 | 15 | 13.9307 | 1.06935 |
| 6 | -1 | 0 | 6.5 | 25 | 13.63 | 13.5189 | 0.11108 |
| 7 | -1 | 0 | 5.5 | 50 | . | 16.4766 | . |
| 8 | -1 | 0 | 6 | 50 | 14.56 | 14.3358 | 0.2242 |
| 9 | -1 | 0 | 6.5 | 50 | 14.34 | 13.6477 | 0.69233 |
| 10 | -1 | 0.56 | 5.5 | 0 | 13.84 | 14.085 | -0.245 |
| 11 | -1 | 0.56 | 6 | 0 | 12.72 | 13.657 | -0.937 |
| 12 | -1 | 0.56 | 6.5 | 0 | 13.08 | 13.5217 | -0.4417 |
| 13 | -1 | 0.56 | 5.5 | 25 | 15.46 | 15.3466 | 0.11344 |
| 14 | -1 | 0.56 | 6 | 25 | 15.58 | 14.0622 | 1.51784 |
| 15 | -1 | 0.56 | 6.5 | 25 | 15.31 | 13.6504 | 1.65957 |
| 16 | -1 | 0.56 | 5.5 | 50 | . | 16.6081 | . |
| 17 | -1 | 0.56 | 6 | 50 | 15.16 | 14.4673 | 0.69269 |
| 18 | -1 | 0.56 | 6.5 | 50 | 14.61 | 13.7792 | 0.83082 |
| 19 | -1 | 5.55 | 5.5 | 0 | 14.96 | 15.2568 | -0.2968 |
| 20 | -1 | 5.55 | 6 | 0 | 14.99 | 14.8288 | 0.16117 |
| 21 | -1 | 5.55 | 6.5 | 0 | 14.25 | 14.6935 | -0.4435 |
| 22 | -1 | 5.55 | 5.5 | 25 | . | 16.5184 | . |
| 23 | -1 | 5.55 | 6 | 25 | 15.21 | 15.234 | -0.024 |
| 24 | -1 | 5.55 | 6.5 | 25 | 13.92 | 14.8222 | -0.9022 |
| 25 | -1 | 5.55 | 5.5 | 50 | . | 17.7799 | . |
| 26 | -1 | 5.55 | 6 | 50 | 17.88 | 15.6391 | 2.24088 |
| 27 | -1 | 5.55 | 6.5 | 50 | 15.43 | 14.951 | 0.479 |
| 1 | 7 | 0 | 5.5 | 0 | 13.76 | 16.1935 | -2.4335 |
| 2 | 7 | 0 | 6 | 0 | 15.9 | 15.7655 | 0.1345 |
| 3 | 7 | 0 | 6.5 | 0 | 16.68 | 15.6302 | 1.04984 |
| 4 | 7 | 0 | 5.5 | 25 | 16.4 | 17.455 | -1.0551 |
| 5 | 7 | 0 | 6 | 25 | 16.76 | 16.1706 | 0.58935 |
| 6 | 7 | 0 | 6.5 | 25 | 16.03 | 15.7589 | 0.27109 |
| 7 | 7 | 0 | 5.5 | 50 | 17.93 | 18.7166 | -0.7866 |
| 8 | 7 | 0 | 6 | 50 | 14.78 | 16.5758 | -1.7958 |
| 9 | 7 | 0 | 6.5 | 50 | 14.46 | 15.8877 | -1.4277 |
| 10 | 7 | 0.56 | 5.5 | 0 | 18.07 | 16.4225 | 1.64755 |
| 11 | 7 | 0.56 | 6 | 0 | 17.2 | 15.9945 | 1.20552 |
| 12 | 7 | 0.56 | 6.5 | 0 | 16.78 | 15.8591 | 0.92086 |
| 13 | 7 | 0.56 | 5.5 | 25 | 15.31 | 17.684 | -2.374 |
| 14 | 7 | 0.56 | 6 | 25 | 17 | 16.3996 | 0.60038 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| 15 | 7 | 0.56 | 6.5 | 25 | 16.49 | 15.9879 | 0.50211 |
| 16 | 7 | 0.56 | 5.5 | 50 | 13.15 | 18.9456 | -5.7956 |
| 17 | 7 | 0.56 | 6 | 50 | 16.01 | 16.8048 | -0.7948 |
| 18 | 7 | 0.56 | 6.5 | 50 | 14.8 | 16.1166 | -1.3166 |
| 19 | 7 | 5.55 | 5.5 | 0 | 19.92 | 18.4628 | 1.45721 |
| 20 | 7 | 5.55 | 6 | 0 | 19.12 | 18.0348 | 1.08519 |
| 21 | 7 | 5.55 | 6.5 | 0 | 15.85 | 17.8995 | -2.0495 |
| 22 | 7 | 5.55 | 5.5 | 25 | 17.99 | 19.7244 | -1.7344 |
| 23 | 7 | 5.55 | 6 | 25 | 18.59 | 18.44 | 0.15004 |
| 24 | 7 | 5.55 | 6.5 | 25 | 17.53 | 18.0282 | -0.4982 |
| 25 | 7 | 5.55 | 5.5 | 50 | 22.51 | 20.9859 | 1.52407 |
| 26 | 7 | 5.55 | 6 | 50 | 17.06 | 18.8451 | -1.7851 |
| 27 | 7 | 5.55 | 6.5 | 50 | 17.48 | 18.157 | -0.677 |
| 1 | 15 | 0 | 5.5 | 0 | 18.22 | 18.4335 | -0.2135 |
| 2 | 15 | 0 | 6 | 0 | 18.03 | 18.0055 | 0.02451 |
| 3 | 15 | 0 | 6.5 | 0 | 16.62 | 17.8702 | -1.2502 |
| 4 | 15 | 0 | 5.5 | 25 | 20.34 | 19.695 | 0.64496 |
| 5 | 15 | 0 | 6 | 25 | 18.84 | 18.4106 | 0.42936 |
| 6 | 15 | 0 | 6.5 | 25 | 17.26 | 17.9989 | -0.7389 |
| 7 | 15 | 0 | 5.5 | 50 | 19.52 | 20.9566 | -1.4366 |
| 8 | 15 | 0 | 6 | 50 | 18.25 | 18.8158 | -0.5658 |
| 9 | 15 | 0 | 6.5 | 50 | 17.32 | 18.1277 | -0.8077 |
| 10 | 15 | 0.56 | 5.5 | 0 | 19.98 | 18.7599 | 1.22008 |
| 11 | 15 | 0.56 | 6 | 0 | 17.87 | 18.3319 | -0.4619 |
| 12 | 15 | 0.56 | 6.5 | 0 | 19.47 | 18.1966 | 1.2734 |
| 13 | 15 | 0.56 | 5.5 | 25 | 21.57 | 20.0215 | 1.54851 |
| 14 | 15 | 0.56 | 6 | 25 | 19.48 | 18.7371 | 0.74291 |
| 15 | 15 | 0.56 | 6.5 | 25 | 18.99 | 18.3254 | 0.66465 |
| 16 | 15 | 0.56 | 5.5 | 50 | 25.73 | 21.2831 | 4.44695 |
| 17 | 15 | 0.56 | 6 | 50 | 19.88 | 19.1422 | 0.73777 |
| 18 | 15 | 0.56 | 6.5 | 50 | 18.37 | 18.4541 | -0.0841 |
| 19 | 15 | 5.55 | 5.5 | 0 | 20.86 | 21.6688 | -0.8088 |
| 20 | 15 | 5.55 | 6 | 0 | 20.59 | 21.2408 | -0.6508 |
| 21 | 15 | 5.55 | 6.5 | 0 | 22.05 | 21.1055 | 0.94455 |
| 22 | 15 | 5.55 | 5.5 | 25 | 23.21 | 22.9303 | 0.27966 |
| 23 | 15 | 5.55 | 6 | 25 | 22 | 21.6459 | 0.35406 |
| 24 | 15 | 5.55 | 6.5 | 25 | 19.82 | 21.2342 | -1.4142 |
| 25 | 15 | 5.55 | 5.5 | 50 | 27.36 | 24.1919 | 3.16809 |
| 26 | 15 | 5.55 | 6 | 50 | 22.16 | 22.0511 | 0.10891 |
| 27 | 15 | 5.55 | 6.5 | 50 | 19.9 | 21.363 | -1.463 |

Note: '.', missing data.

**TABLE B4** Model prediction residuals for -1–15$^{o}$C and anaerobic conditions.

| Medium (#) | T ($^{o}$C) | Glucose (mM) | pH | LA (mM) | Diameter of inhibition zone (mm) | | |
|---|---|---|---|---|---|---|---|
| | | | | | Observation | Prediction | Residual |
| 1 | -1 | 0 | 5.5 | 0 | 16.39 | 13.2343 | 3.15569 |
| 2 | -1 | 0 | 6 | 0 | 13.89 | 13.2343 | 0.65569 |
| 3 | -1 | 0 | 6.5 | 0 | 13.94 | 13.2343 | 0.70569 |
| 4 | -1 | 0 | 5.5 | 25 | 13.19 | 13.2343 | -0.0443 |
| 5 | -1 | 0 | 6 | 25 | 13.77 | 13.2343 | 0.53569 |
| 6 | -1 | 0 | 6.5 | 25 | 13.36 | 13.2343 | 0.12569 |
| 7 | -1 | 0 | 5.5 | 50 | . | 13.2343 | . |
| 8 | -1 | 0 | 6 | 50 | 13.86 | 13.2343 | 0.62569 |
| 9 | -1 | 0 | 6.5 | 50 | 14.81 | 13.2343 | 1.57569 |
| 10 | -1 | 0.56 | 5.5 | 0 | 13.43 | 13.2419 | 0.18814 |
| 11 | -1 | 0.56 | 6 | 0 | 13.93 | 13.2419 | 0.68814 |
| 12 | -1 | 0.56 | 6.5 | 0 | 13.37 | 13.2419 | 0.12814 |
| 13 | -1 | 0.56 | 5.5 | 25 | 12.37 | 13.2419 | -0.8719 |
| 14 | -1 | 0.56 | 6 | 25 | 14.43 | 13.2419 | 1.18814 |
| 15 | -1 | 0.56 | 6.5 | 25 | 14.24 | 13.2419 | 0.99814 |
| 16 | -1 | 0.56 | 5.5 | 50 | 14.35 | 13.2419 | 1.10814 |
| 17 | -1 | 0.56 | 6 | 50 | 14.01 | 13.2419 | 0.76814 |
| 18 | -1 | 0.56 | 6.5 | 50 | 13.87 | 13.2419 | 0.62814 |
| 19 | -1 | 5.55 | 5.5 | 0 | 15.15 | 13.3091 | 1.84086 |
| 20 | -1 | 5.55 | 6 | 0 | 12.63 | 13.3091 | -0.6791 |
| 21 | -1 | 5.55 | 6.5 | 0 | 14.77 | 13.3091 | 1.46086 |
| 22 | -1 | 5.55 | 5.5 | 25 | 18.09 | 13.3091 | 4.78086 |
| 23 | -1 | 5.55 | 6 | 25 | 13.92 | 13.3091 | 0.61086 |
| 24 | -1 | 5.55 | 6.5 | 25 | 13.56 | 13.3091 | 0.25086 |
| 25 | -1 | 5.55 | 5.5 | 50 | . | 13.3091 | . |
| 26 | -1 | 5.55 | 6 | 50 | 14.15 | 13.3091 | 0.84086 |
| 27 | -1 | 5.55 | 6.5 | 50 | 14.72 | 13.3091 | 1.41086 |
| 1 | 7 | 0 | 5.5 | 0 | 13.57 | 15.7863 | -2.2163 |
| 2 | 7 | 0 | 6 | 0 | 14.27 | 15.7863 | -1.5163 |
| 3 | 7 | 0 | 6.5 | 0 | 16.91 | 15.7863 | 1.12372 |
| 4 | 7 | 0 | 5.5 | 25 | 13.42 | 15.7863 | -2.3663 |
| 5 | 7 | 0 | 6 | 25 | 16.51 | 15.7863 | 0.72372 |
| 6 | 7 | 0 | 6.5 | 25 | 13.9 | 15.7863 | -1.8863 |
| 7 | 7 | 0 | 5.5 | 50 | 15.12 | 15.7863 | -0.6663 |
| 8 | 7 | 0 | 6 | 50 | 12.92 | 15.7863 | -2.8663 |
| 9 | 7 | 0 | 6.5 | 50 | 14.22 | 15.7863 | -1.5663 |
| 10 | 7 | 0.56 | 5.5 | 0 | 14.82 | 15.9185 | -1.0985 |
| 11 | 7 | 0.56 | 6 | 0 | 15.16 | 15.9185 | -0.7585 |
| 12 | 7 | 0.56 | 6.5 | 0 | 15.33 | 15.9185 | -0.5885 |
| 13 | 7 | 0.56 | 5.5 | 25 | 13.2 | 15.9185 | -2.7185 |
| 14 | 7 | 0.56 | 6 | 25 | 15.25 | 15.9185 | -0.6685 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| 15 | 7 | 0.56 | 6.5 | 25 | 13.52 | 15.9185 | -2.3985 |
| 16 | 7 | 0.56 | 5.5 | 50 | 14.05 | 15.9185 | -1.8685 |
| 17 | 7 | 0.56 | 6 | 50 | 14.78 | 15.9185 | -1.1385 |
| 18 | 7 | 0.56 | 6.5 | 50 | 13.69 | 15.9185 | -2.2285 |
| 19 | 7 | 5.55 | 5.5 | 0 | 14.22 | 17.0965 | -2.8765 |
| 20 | 7 | 5.55 | 6 | 0 | 15.62 | 17.0965 | -1.4765 |
| 21 | 7 | 5.55 | 6.5 | 0 | 13.4 | 17.0965 | -3.6965 |
| 22 | 7 | 5.55 | 5.5 | 25 | 14.17 | 17.0965 | -2.9265 |
| 23 | 7 | 5.55 | 6 | 25 | 14.95 | 17.0965 | -2.1465 |
| 24 | 7 | 5.55 | 6.5 | 25 | 15.37 | 17.0965 | -1.7265 |
| 25 | 7 | 5.55 | 5.5 | 50 | 16.09 | 17.0965 | -1.0065 |
| 26 | 7 | 5.55 | 6 | 50 | 14.48 | 17.0965 | -2.6165 |
| 27 | 7 | 5.55 | 6.5 | 50 | 14.92 | 17.0965 | -2.1765 |
| 1 | 15 | 0 | 5.5 | 0 | 18 | 18.3382 | -0.3383 |
| 2 | 15 | 0 | 6 | 0 | 17.53 | 18.3382 | -0.8083 |
| 3 | 15 | 0 | 6.5 | 0 | 17.47 | 18.3382 | -0.8683 |
| 4 | 15 | 0 | 5.5 | 25 | 19.05 | 18.3382 | 0.71175 |
| 5 | 15 | 0 | 6 | 25 | 18.67 | 18.3382 | 0.33175 |
| 6 | 15 | 0 | 6.5 | 25 | 17.27 | 18.3382 | -1.0683 |
| 7 | 15 | 0 | 5.5 | 50 | 22.87 | 18.3382 | 4.53175 |
| 8 | 15 | 0 | 6 | 50 | 18.62 | 18.3382 | 0.28175 |
| 9 | 15 | 0 | 6.5 | 50 | 17.81 | 18.3382 | -0.5283 |
| 10 | 15 | 0.56 | 5.5 | 0 | 18.5 | 18.5951 | -0.0951 |
| 11 | 15 | 0.56 | 6 | 0 | 18.01 | 18.5951 | -0.5851 |
| 12 | 15 | 0.56 | 6.5 | 0 | 18.47 | 18.5951 | -0.1251 |
| 13 | 15 | 0.56 | 5.5 | 25 | 21.78 | 18.5951 | 3.1849 |
| 14 | 15 | 0.56 | 6 | 25 | 20.13 | 18.5951 | 1.5349 |
| 15 | 15 | 0.56 | 6.5 | 25 | 19.31 | 18.5951 | 0.7149 |
| 16 | 15 | 0.56 | 5.5 | 50 | 22.8 | 18.5951 | 4.2049 |
| 17 | 15 | 0.56 | 6 | 50 | 20.01 | 18.5951 | 1.4149 |
| 18 | 15 | 0.56 | 6.5 | 50 | 18.83 | 18.5951 | 0.2349 |
| 19 | 15 | 5.55 | 5.5 | 0 | 23.03 | 20.8838 | 2.14619 |
| 20 | 15 | 5.55 | 6 | 0 | 20.59 | 20.8838 | -0.2938 |
| 21 | 15 | 5.55 | 6.5 | 0 | 20.99 | 20.8838 | 0.10619 |
| 22 | 15 | 5.55 | 5.5 | 25 | 23.7 | 20.8838 | 2.81619 |
| 23 | 15 | 5.55 | 6 | 25 | 22.91 | 20.8838 | 2.02619 |
| 24 | 15 | 5.55 | 6.5 | 25 | 19.95 | 20.8838 | -0.9338 |
| 25 | 15 | 5.55 | 5.5 | 50 | 24.11 | 20.8838 | 3.22619 |
| 26 | 15 | 5.55 | 6 | 50 | 22.19 | 20.8838 | 1.30619 |
| 27 | 15 | 5.55 | 6.5 | 50 | 20.43 | 20.8838 | -0.4538 |

Note: ‘.’, missing data.

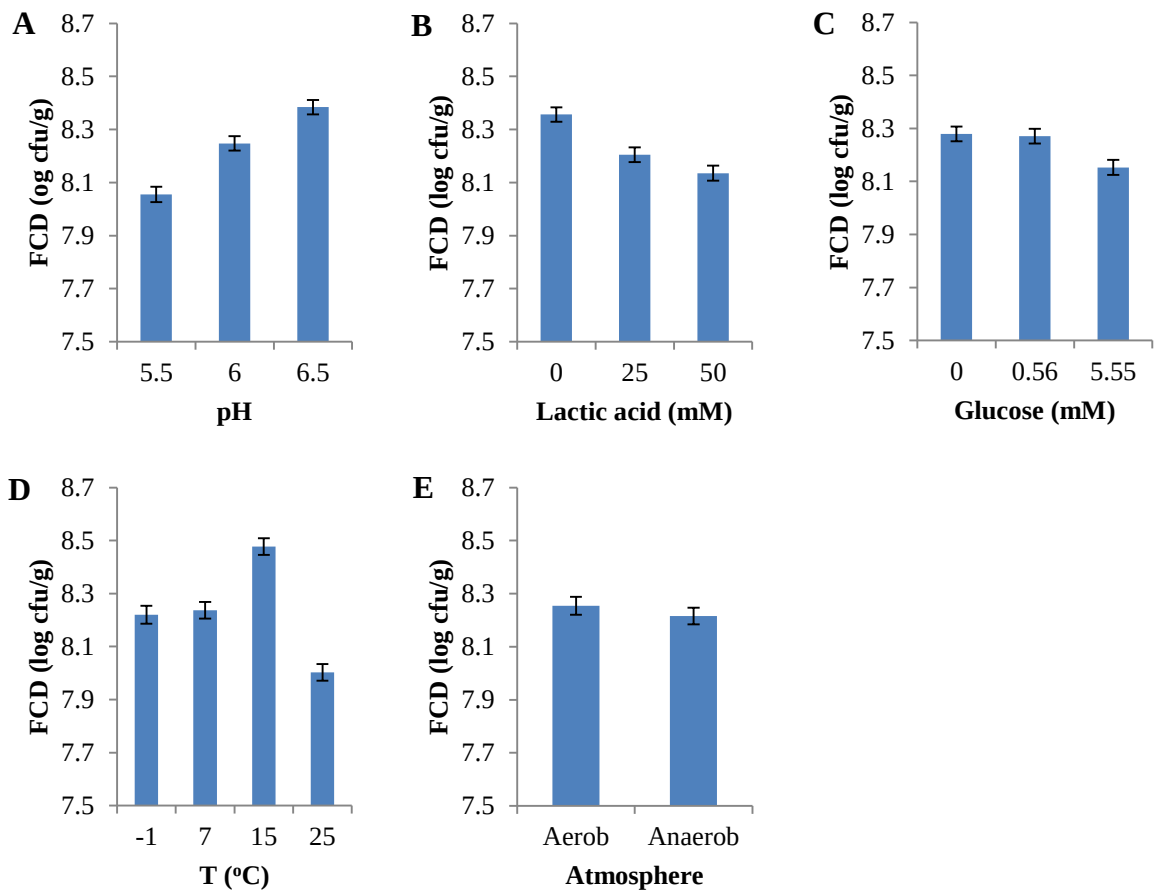



**FIG B1** Effect of environmental factors on final cell density (FCD).

The effect was significant (F–test, $P < 0.05$) for the factors temperature, glucose, lactic acid, and pH (A, B, C, and D), but not for atmosphere (E). Error bars represent standard error of the mean. In this assay, a small piece (approximately 0.5 g) of agar was sampled from an area of mBHI agar not displaying an inhibition zone. Agar was weighed, pulverized, and then mixed with peptone water (bacteriological peptone 1 g/L, NaCl 8.5 g/L, pH 7.3 ± 0.2). CFU were determined by plate-count.

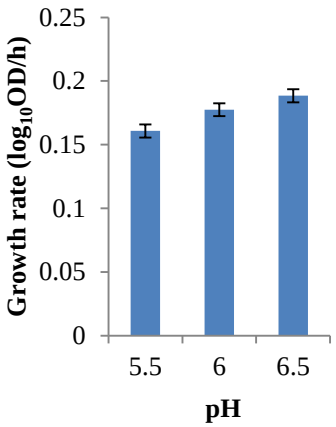



**FIG B2** Effect of pH on growth rate of *Carnobacterium maltaromaticum* D8c.

Error bars represent standard error of the mean.

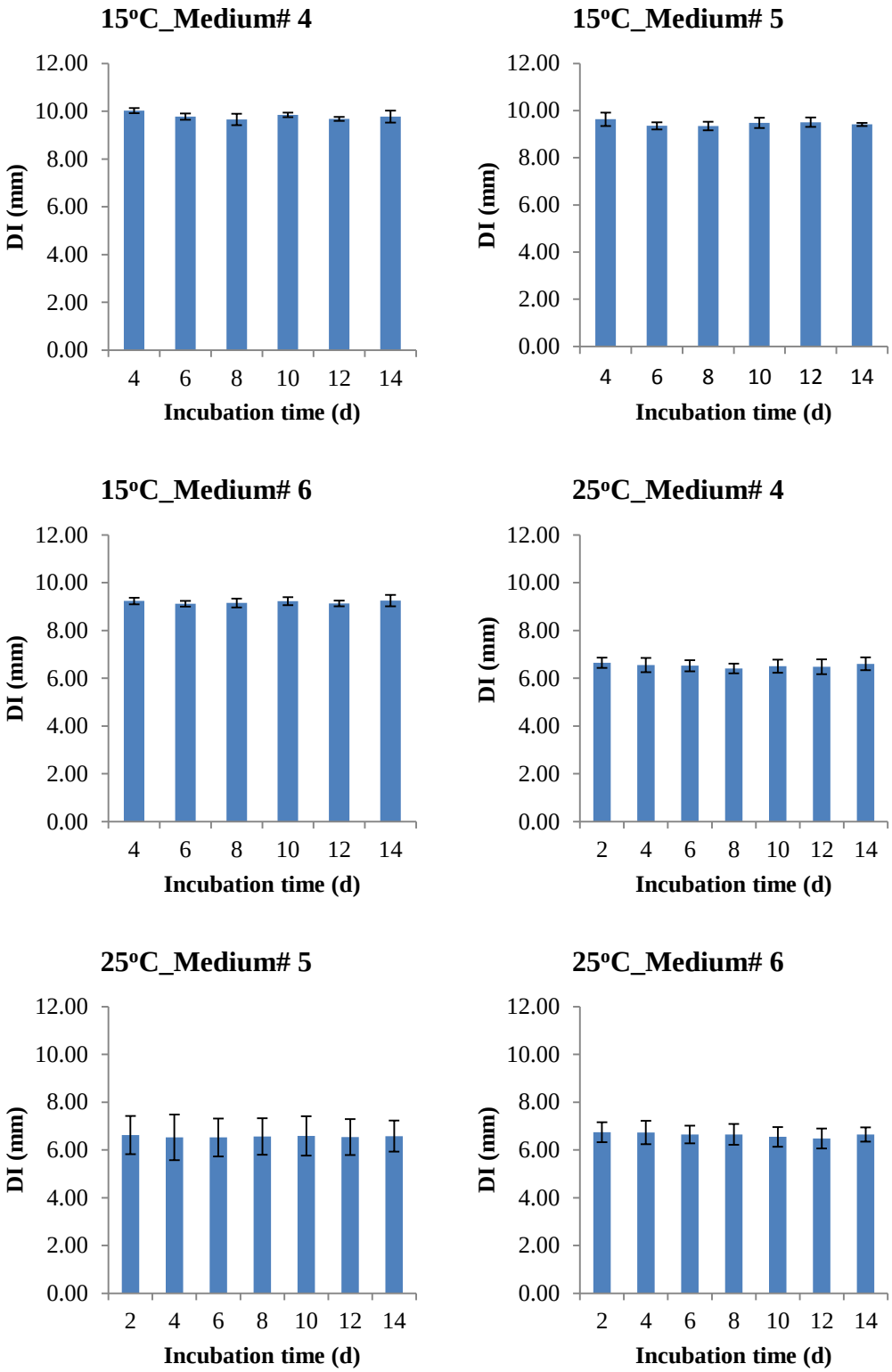



**FIG B3** Effect of incubation time on diameter of inhibition zone (DI).

Error bars represent standard deviation.

| Filtration unit cutoff (KDa) | 3 | 3 | 10 | 10 | 50 | 50 | 100 | 100 |
|---|---|---|---|---|---|---|---|---|
| Filtrate (F) / Retentate (R) | F | R | F | R | F | R | F | R |
| Inhibition shown (+) /not shown (-) | - | + | - | + | - | - | - | - |

**FIG B4** Inhibitory activity of *Carnobacterium maltaromaticum* D0h CFS after ultrafiltration

Cell-free supernatant (CFS) produced by *C. maltaromaticum* D0h were filtered through ultrafiltration units with cut-offs of 3, 10, 50, and 100 KDa, respectively. The inhibitory activity of filtrates (F) and retentates (R) were measured via agar overlay method as described in *MATERIALS AND METHODS: Measurement of CFS inhibitory activity* in Chapter 5.

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