Inter-strain Interactions between Bacteria Isolated from Vacuum-Packaged Refrigerated Beef

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Running title: Interaction among beef bacterial isolates

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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 twenty target isolates were selected, representing ten 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 sixteen 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 (1). 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 (2-4). 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 (5).

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 (5, 6). However, the underlying forces of microbial interactions may also be important in shaping biodiversity of communities (7-10); 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 (10-14). There have been
numerous reports exploring the effectiveness of protective cultures and related antibacterial compounds at enhancing food safety and extending shelf-life (15-18), however few have investigated interactions among food bacteria, and of those which have, relatively few species have been studied (19-22); far fewer have involved species from diverse communities (7, 23).

Nychas et al. (24) found quorum-sensing compounds extracted from meat increased the growth rate of *Serratia marcescens* and *Pseudomonas fluorescens*. Also, Russo et al. (19) 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 (25) has been widely used to detect bacterial inhibitory activity, via reporting an inhibition zone (8, 26, 27). 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 (24).

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. (28). 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.

In this study, the 180 isolates were screened for inhibitory activity, using a spot-lawn method (25) 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 & 2). Six (*Leuconostoc mesenteroides* B30b, *Staphylococcus epidermidis* F30c, *Bacillus* sp. 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 to have a panel of isolates representing those species found in VP beef from different abattoirs. Isolates were stored at -80°C in brain heart infusion broth (BHI; Amyl Media Ltd, Australia), supplemented with 20% (vol/vol) glycerol.

**Inhibition activity measured on agar.** The spot-lawn method described by Benkerroum et al. (25) 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 OD<sub>540nm</sub> 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 spotted on the target lawn. Inhibition was measured after 24 h incubation at 25°C, when TSA plates were photographed and the diameter (D) of the inhibition zone 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 ≥ 4 mm, 2 ≤ D < 4 mm, 0.5 < D < 2 mm and 0 < D ≤ 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.

**Interaction activity measured by cell-free supernatant (CFS) assay.** Overnight cultures (24 h, 25°C) of target isolates were adjusted to 10^4 cfu/ml. Effector isolates were incubated for 48 h at 25°C until the stationary phase was reached. CFS of each effector isolates were made by centrifuging BHI cultures at 1000 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 PBS, instead of CFS (1M, pH 7.4).

Duplicate wells were used for all treatments and controls. BioscreenC temperature was 25°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; h) was calculated as the time to reach an OD_{540nm} of 0.12 (background corrected data). The Baranyi model (29) was fitted to the primary growth curves using DMFit (version 3.0; [http://www.combase.cc/index.php/en/downloads/category/11-dmfit]) to calculate growth rate (GR; log_{10}OD/h). Maximum population density (MPD; OD_{540nm})
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 \( p < 0.05 \), IS was calculated by comparison of treatment and PBS control using the following formulas:

\[
\begin{align*}
\text{IS}_{DT} &= \frac{|DT_{\text{Treatment}} - DT_{\text{Control}}|}{DT_{\text{Control}}} \\
\text{IS}_{GR} &= \frac{|GR_{\text{Treatment}} - GR_{\text{Control}}|}{GR_{\text{Control}}} \\
\text{IS}_{MPD} &= \frac{|MPD_{\text{Treatment}} - MPD_{\text{Control}}|}{MPD_{\text{Control}}}
\end{align*}
\]

Cumulative IS effect (IS\(_{\text{Total}}\)) on all three growth parameters was quantified using the formula:

\[
\text{IS}_{\text{Total}} = \frac{(\text{IS}_{DT} + \text{IS}_{GR} + \text{IS}_{MPD})}{3}
\]

Promotion strength (PS) was calculated similar to IS, via comparison of test and BHI control. IS was classified at four levels: ++++, +++, ++, +, corresponding to IS = 1 (no detectable growth of the target), \( 0.25 \leq \text{IS} < 1 \), \( 0.15 \leq \text{IS} < 0.25 \), and \( 0 \leq \text{IS} < 0.15 \), respectively (Fig. 2).
the relatively fewer instances where CFS promoted growth, growth promotion strength (PS) was classified at two levels: ++ and +, corresponding to $PS \geq 0.1$ and $0 < PS < 0.1$, respectively.

Network maps of bacterial interactions. Growth inhibition/promotion activity was described using a network diagram drawn with Cytoscape (version 3.1.1; [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 (Eq. 5).

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

with $i$ being the interaction level (1, +; 2, ++; 3, +++; 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.

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. F-test was applied to examine overall differences among different groups. If the F-test
was significant ($p < 0.05$), the student’s 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 t-test was considered statistically significant.
These tests were performed using the GLM procedure in SAS (version 9.3; [http://www.sas.com]).

RESULTS
A total of 774 and 735 combinations of effector and target isolates were tested by spot-lawn and CFS assay, respectively. The difference of 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).

**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; 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* (data not shown). On the whole, more inhibition events were associated with increased DT (78.9% of inhibitions) than decreased GR (44.7%) and MPD (28.5%).

**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, nine were strong (++)
and 22 weak (+) (Table 3). *Pseudomonas* and Enterobacteriaceae were the most common
growth-promoting effector isolates; less common effectors included *Bacillus* sp. 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. 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).

While most growth-promoting activity reduced DT and/or increased GR (data not shown),
MPD was enhanced in some interactions. For example, *Bacillus subtilis* E0g increased the MPD
of *Pseudomonas* sp. D8g by 0.45 OD_{540nm} units. Similarly, *Serratia* sp. E8c increased the MPD
of *Pseudomonas* sp. D0g by 0.35 OD_{540nm} units (data not shown).

**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 five (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.

Intra-species 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
Growth-promotion was target-dependent and restricted to a relatively small number of isolates, i.e. *Carnobacterium* sp. D30f and D8c, *Pseudomonas* sp. 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. A30g and E0g promoted the growth of *Pseudomonas* sp. D8g, displaying PS of 0.15 and 0.32, respectively (data not shown). *Serratia* sp. E8c promoted the growth of both *Pseudomonas* sp. D8g and D0g, at PS 0.37 and 0.12, respectively (data not shown).

**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 1; 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. 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; 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 (9), but 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 (7-9), influencing food quality and safety.

In this 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 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 (57). 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, lactic acid and/or other antibacterial substances (14, 18, 30). In this study, *C. maltaromaticum* isolates inhibited from five (C0a) to 10 (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, while *L. carnosum* inhibited two (Table 1). Interestingly, *C. maltaromaticum* and *C. divergens* also showed strong intra-species inhibition (Fig. 3A and B), consistent with the studies of Martin-
Visscher et al. (30) and Worobo et al. (31). 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 (data not shown). These differences may due to inhibitory factors in CFS, such as disassociated lactic acid and bacteriocins, commonly produced by *Carnobacterium* spp. (32).

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; Table 1). *Pseudomonas* sp. D0b inhibited 18 of the 20 targets (Table 1). Similarly, Aguirre-von-Wobeser et al. (27), 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 (33-35). These have been applied in plant pathology to control fungal pathogens, and in clinical studies to inhibit pathogenic strains (36-38).
However, antibacterial compounds might not explain all the inhibitory activities of \textit{Pseudomonas} spp., since inhibition patterns of \textit{Pseudomonas} spp. differed markedly between spot-lawn and CFS assay. For example, \textit{Pseudomonas} sp. D0b CFS only inhibited one target by CFS, but 17 by spot-lawn. This may indicate live effector cells, not just CFS, are required for target inhibition, as reported by Russell et al. (39) who found \textit{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 \textit{Pseudomonas} spp. differ in solid versus liquid media.

It was also noted that growth of \textit{C. divergens} D30f and \textit{C. maltaromaticum} D8c was promoted by CFS from most \textit{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 \textit{Pseudomonas} spp. on sensitive bacteria, such as \textit{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 \textit{Bacillus} sp. 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 (40). Baindara et al. (41) characterized two antimicrobial peptides produced by a \textit{B.}
Bacillus 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 (42, 43); 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 (12).

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. (44), 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 (8, 26, 27), 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 (45, 46).

In this study, *Leuconostoc* sp. F30e failed to grow in BHI at 25°C, hence 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 from chill-stored foods and may not readily grow at elevated temperature, including 25°C used in this study (47-49).

While the general focus of this study was measuring growth inhibition and promotion, different inhibition zone morphologies were observed on agar, possibly indicating different mechanisms of action. Undefined (diffuse) inhibition zones have been observed in antibiotic resistance studies (50, 51), and interpreted as low levels of bacteria resistance. We noted Pseudomonas spp. often produced such a diffuse type of inhibition zone.

This study 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 these studies used bacteriological broth, relatively high densities of cells, and pair-wise comparison of isolates (7).

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

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<td></td>
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</tr>
<tr>
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<td>19</td>
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<td>0</td>
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<td>19</td>
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TABLE 2 Effectors inhibiting or promoting growth of target isolates

<table>
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<tr>
<th>Target</th>
<th>Isolate code</th>
<th>Inhibition (%)</th>
<th>Promotion (%)</th>
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<td><em>Carnobacterium divergens</em></td>
<td>D30f</td>
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<td><em>Carnobacterium maltaromaticum</em></td>
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<td>25.6</td>
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<tr>
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</tr>
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<td><em>Brochothrix thermosphacta</em></td>
<td>A0b</td>
<td>43.6</td>
<td>5.1</td>
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<tr>
<td><em>Yersinia sp.</em></td>
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<td>25.6</td>
<td>0</td>
</tr>
<tr>
<td><em>Bacillus subtilis</em></td>
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<td><em>Bacillus sp.</em></td>
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<td><em>Serratia sp.</em></td>
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<td><em>Pseudomonas lundensis</em></td>
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<td><em>Staphylococcus epidermidis</em></td>
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<td>0</td>
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<td><em>Rahnella aquatilis</em></td>
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</table>

* Percentages of target isolates where growth was inhibited or promoted.
TABLE 3 Summary of growth-inhibition and -promotion activity

<table>
<thead>
<tr>
<th>Interaction levela</th>
<th>Spot-lawn</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th>CFSb</th>
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<tr>
<td></td>
<td>Total</td>
<td>Inhibition</td>
<td></td>
<td></td>
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<td>Promotionc</td>
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<td>+</td>
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<td>Totald</td>
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<td>16.6</td>
<td>31</td>
<td>4.2</td>
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</tr>
</tbody>
</table>

a For spot-lawn assay, ++++ = D ≥ 4 mm; +++ = 2 mm ≤ D < 4 mm; ++ = 0.5 mm < D < 2 mm, + = 0 < D ≤ 0.5 mm. For 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. For 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 Total number or percentage of effector-target pairings displaying inhibition or promotion among 774 and 735 effector-target parings studied by spot-lawn and CFS assay, respectively.
TABLE 4  Effects on growth parameters measured by CFS assay

<table>
<thead>
<tr>
<th></th>
<th>Inhibition</th>
<th>Promotion</th>
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<tr>
<td>%</td>
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<td>%</td>
</tr>
<tr>
<td>DT</td>
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<td>GR</td>
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<td>MPD</td>
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</tbody>
</table>

*DT = detection time, GR = growth rate, MPD = maximum population density

Percentage was based on the number of interactions affecting a specific growth parameter, divided the total number of interactions (inhibition, 122; promotion, 31).

LIST OF FIGURES

Figure 1. Representative growth-inhibition by spot-lawn assay.

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

Figure 3. Interactions among effector and target isolates.
Figure 1. Representative growth-inhibition by spot-lawn assay.

Inhibition of target isolates designated 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.

Figure 2. Representative growth-inhibition and -promotion by CFS-broth assay.
Figure 3. Interactions among effector and target isolates.

A, Inhibition: spot-lawn assay; B, Inhibition: CFS assay; C, Promotion: CFS assay

- \( \Diamond \) = target; \( \bigcirc \) = effector; \( \square \) = isolate tested as both target and effector. \( a \rightarrow b \) = a inhibited (A & B) or promoted b (C).

- \( \rightarrow \) or \( \overrightarrow{\text{---}} \) = ++++ inhibition.
- \( \rightarrow \) or \( \overrightarrow{\text{---}} \) = +++ inhibition.

- \( \rightarrow \) or \( \overrightarrow{\text{---}} \) = ++ inhibition.
- \( \rightarrow \) or \( \overrightarrow{\text{---}} \) = + inhibition.

and \( \rightarrow \) = ++ and +

growth promotion, respectively. \( \overrightarrow{\text{---}} \) and \( \overrightarrow{\text{---}} \) = diffuse and clear inhibition zones (A). In 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 C, the size of both an effector and target node is
positively correlated with the number and level of promotions.