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Diversity of universal stress protein in Enterobacteriales and its reduced expressions on *Pectobacterium brasiliense* after manuka honey treatment

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Abstract. *Chotimah, Soffan A, Joko T. 2024. Diversity of universal stress protein in Enterobacteriales and its reduced expressions on* Pectobacterium brasiliense *after manuka honey treatment. Biodiversitas 25: 49-52.* Universal stress proteins (Usps) support the survival of an organism by increasing their expressions under stress conditions, which include nutritional deficiencies, heat shock, and antibiotics. However, the expression of *uspA* in *Staphylococcus aureus* decreased after manuka honey exposure. Manuka honey also inhibits the growth of *Pectobacterium brasiliense*, although its effect on the *usp* gene of this soft-rot pathogen has never been studied. This study aimed to determine the diversity of Usps in *P. brasiliense* and their orthologs from Enterobacteriales using phylogenetic analysis. The effects of manuka honey on *usp* gene expressions were also investigated using quantitative real-time polymerase chain reaction and pathogenicity assay of *P. brasiliense* on Chinese cabbage. The results revealed that the UspA, UspB, and UspE of *P. brasiliense* had the closest similarity to those of *P. carotovorum.* By contrast, UspG had the closest similarity to *P. polaris*. The *usp* gene expressions were downregulated by 5, 16, 10, and 62% in 5% (w/v) manuka honey treatment. When tested on Chinese cabbage, *P. brasiliense* treated with manuka honey caused smaller lesion symptoms than those in the control treatment. This reduced virulence of *P. brasiliense* may be related to the reduced expression of *usp* genes triggered by manuka honey.

Keywords: Down-regulation, pathogen, soft-rot, q-RT-PCR, virulence

INTRODUCTION

The genus *Pectobacterium* is a member of soft-rot Pectobacteriaceae, which includes opportunistic phytopathogens that can switch from an asymptomatic latent phase into a virulent phase under favorable environmental conditions, either in the field or during storage (Loc et al. 2022). In the last few decades, the taxonomy of *Pectobacterium* has undergone major modifications and reclassifications. A total of 22 species have been validly reported: *P. actinidiae* (Portier et al. 2019), *P. aquaticum* (Pédron et al. 2019), *P. aroidearum* (Xu et al. 2021), *P. atrosepticum* (Ismiyatuningsih et al. 2016; Toth et al. 2022), *P. betavasculorum* (Rastgou et al. 2022), *P. brasiliense* (Oulghazi et al. 2021), *P. cacticida* (Xu et al. 2021), *P. carotovorum* (Portier et al. 2019), *P. colocasium* (Zhou et al. 2022), *P. fontis* (Oulghazi et al. 2019), *P. jejuense* (Hong et al. 2023), *P. odoriferum* (Jin et al. 2022), *P. parmentieri* (Khayi et al. 2016), *P. parvum* (Pasanen et al. 2020), *P. peruviense* (Waleron et al. 2018), *P. polaris* (Dees et al. 2017), *P. polonicum* (Waleron et al. 2019a), *P. punjabense* (Sarfraz et al. 2018), *P. quasiaquaticum* (Moussa et al. 2021), *P. versatile* (Kravchenko et al. 2021), *P. wasabiae* (Khayi et al. 2016), and *P. zantedeschiae* (Waleron et al. 2019b). Among these species, *Pectobacterium brasiliense* is considered the most virulent and highly aggressive soft-rot pathogen.

Since the discovery of universal stress protein A (UspA) in *Escherichia coli* (Nyström and Neidhardt 1992), several studies have identified *usp* genes as conserved genes in several bacterial organisms including archaea, plants, and invertebrates. The Usp plays a role in the adaptation of organisms to external stresses, such as genotoxicity, heat shock, membrane damage, and nutrient starvation (Vollmer and Bark 2018). Several bacterial Usps, which are involved in cell growth under stress conditions, biofilm formation, motility, and virulence, have been studied (Ye et al. 2020). The *uspA*-like genes in *Treponema denticola* and *Porphyromonas gingivalis* play a role in biofilm formation (Chopra et al. 2020). The Usps in *Salmonella enterica* participate in biofilm formation in response to benzalkonium chloride (Obe et al. 2021).

To date, the role of Usps in plant-pathogenic bacteria, including the soft-rot pathogen *P. brasiliense*, remains unknown (Joko et al. 2018; Charkowski 2018). *P. brasiliense* causes severe damage to many hosts, especially solanaceous plants (Oulghazi et al. 2020). Antibiotics, which are prone to cause resistance, are generally used on soft-rot bacteria; therefore, other environmentally safe compounds are needed to control *P. brasiliense*. Manuka honey has antimicrobial activity and the potential to be used as an alternative to antibiotics (Roberts et al. 2015).

In a previous report, manuka honey reduced bacterial virulence by downregulating the *uspA* expression in methicillin-resistant *Staphylococcus aureus* (MRSA) (Jenkins et al. 2011). This downregulation was observed via two-dimensional electrophoresis combined with matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. Interestingly, *usp* genes, whose expressions increase under stress conditions, experienced decreased

expressions. The downregulation of *uspA* was confirmed by real-time polymerase chain reaction (RT-PCR), which showed a 16-fold downregulation of *uspA* under manuka honey treatment. The decrease in *uspA* expression in MRSA was caused by the bacterium's inability to accommodate the stress triggered by manuka honey (Jenkins et al. 2014).

Ava et al. (2022) reported that manuka honey can also reduce the virulence of *P. brasiliense* in *Dendrobium* orchid by suppressing genes encoding plant cell wall degrading enzymes (PWDCEs). Perhaps this inhibition caused by manuka honey may not only suppress genes encoding PWDCE in *P. brasiliense* but also affect complex regulatory networks, including *usp* genes. Some studies proved that *usp* genes are related to virulence (Elhosseiny et al. 2015). In this paper, the role of Usps in *P. brasiliense* and their diversity in other bacteria within Enterobacteriales were described through phylogenetic analysis. The virulence of *P. brasiliense* in Chinese cabbage after manuka honey treatment was determined, followed by observation of the effect of manuka honey on *usp* expression using quantitative RT-PCR (qRT-PCR).

MATERIALS AND METHODS

Bacterial strain and growth condition

The study was conducted at the Laboratory of Plant Pathology, Department of Plant Protection, Faculty of Agriculture, Universitas Gadjah Mada, Yogyakarta, Indonesia. The *P. brasiliense* Pal3.4 used in this study was grown on yeast peptone agar medium (0.5% yeast extract, 1% peptone, and 1.5% agar). The bacterial cultures were incubated for 1-2 days at room temperature (±27° C), and single colonies were cultivated regularly in new media to ensure bacterial viability and the purity of isolates (Joko et al. 2019).

Pathogenicity assay of *Pectobacterium brasiliense* on Chinese cabbage

The 24 h-old cultures of *P. brasiliense* were suspended in 5 mL sterile water to OD_{600nm}~0.2 (10^8 cells/mL). Manuka honey (5% w/v) was added to the honey treatment suspension, whereas honey was not added to the control treatment. The Chinese cabbage leaf (*Brassica pekinensis* L.) obtained from a local supermarket was injured with a sterile scalpel blade and then inoculated with 10 µL suspensions of pathogen and control treatments. Incubation was carried out in a closed and moist container. Lesions were observed for up to 36 h post-inoculation (hpi), with the detection of rotten tissue at the point of inoculation at 24 and 36 hpi (Lee et al. 2021).

Phylogenetic analysis of Usps

Phylogenetic analysis was performed by data mining on GenBank (Fauziah and Joko 2021). Four Usps (UspA, UspB, UspE, and UspG) of *P. brasiliense* were used as queries to search for orthologs through BLASTn in National Center for Biotechnology Information (NCBI) (http://blast.ncbi.nlm.nih.gov). The following Usp sequences of *P. brasiliense* were used as queries: UspA and UspB (SRR15733537) from previous transcriptomic research and UspE (WP_010274968.1) and UspG (WP_010276079.1) from the data search on NCBI. Phylogenetic tree construction was carried out using the Maximum-Likelihood (ML) method with the Le-Gascuel (LG) model (Le and Gascuel 2008) based on the Bayesian information criterion (BIC) (Heo et al. 2020) and corrected Akaike information criterion (AICc) score (Sibeijn and Pequito 2022) using MEGA 11software (Tamura et al. 2021).

RNA isolation and cDNA synthesis

Pectobacterium brasiliense was cultured in yeast peptone broth for 12 h for the control and 5% (w/v) manuka honey treatments. RNA isolation was performed using the GENEzol™ Reagent (Geneaid, Taiwan) in accordance with the manufacturer's instructions. The cDNA was synthesized using ReverTra Ace-α-® (TOYOBO) Japan kit on BioRad T100TM Thermal Cycle (Navitasari et al. 2020). The total volume of reaction used was 20 µL, which consisted of 1 µL RNA, 1 µL ReverTra Ace® enzyme, 4 µL 5x buffer, 2 µL dNTP mixture, 1 µL oligo (dT)20, and 10 µL ddH_2O. Incubation was carried out for 20 min at 42° C and heating at 99° C for 5 min.

Analysis of *usp* gene expression

The effect of manuka honey on the *usp* gene expression in *P. brasiliense* was determined through expression analysis. The *usp* expressions in *P. brasiliense* cells treated with 5% (w/v) manuka honey and control were quantified. The expressions of four *usp* genes in *P. brasiliense*, namely, *uspA*, *uspB*, *uspE*, and *uspG*, were analyzed. *recA* was used as the internal standard for level expression analysis. Primers were designed using Primer3Plus (https://primer3plus.com/cgibin/dev/primer3plus.cgi) (Table 1) based on the whole-genome sequences of *P. brasiliense* type strain LMG 21371^T retrieved from the GenBank nucleotide database (https://ncbi.nlm.nih.gov). The annealing temperature of each primer was optimized using a gradient thermal cycler (Biorad T100TM, Germany).

Quantitative analysis was performed using qRT-PCR (BioRad) with THUNDERBIRD® SYBR® qPCR Mix (TOYOBO) and the *recA* gene as an internal standard. After the cDNA synthesis process, each cDNA sample for the treatment and control was homogenized with 2 µL DW reagent, 1 µL forward primer, 1 µL reverse primer, 5 µL THUNDERBIRD®SYBR® qPCR Mix, and 1 µL cDNA into the RT-qPCR microtube and then centrifuged. A total of 40 cycles were conducted under the following conditions: predenaturation at 95° C for 3 min, denaturation at 95° C for 10 s, annealing at 60° C for 15 min and then melting at 95, 65, and 95° C for 30 s with one biological replication and three technical replications. Gene expressions were evaluated by comparing Ct values between treated and nontreated cells using the $2^{-\Delta\Delta Ct}$ method (Widyaningsih et al. 2019).

RESULTS AND DISCUSSION

Pathogenicity assay of *Pectobacterium brasiliense* on Chinese cabbage

A pathogenicity assay was carried out to determine the effect of manuka honey on the host infected with *P. brasiliense*. The result shows that the Chinese cabbage leaf treated with 5% (w/v) manuka honey exhibited smaller lesion symptoms at 24 (Figure 1.A) and 36 hpi (Figure 1.B) compared with that under the control treatment, as indicated by the different lesion sizes observed. A soft, watery, and small brown lesion appeared on the leaf. The lesion was ovoid and elongated in the direction of the leaf vein. The lesion appeared as a small spot at 12 hpi and grew larger with the length of incubation.

The reduction in symptom size under manuka honey treatment was consistent at 24 and 36 hpi. Although the lesion was still enlarged at 36 hpi, the suppression of symptoms by manuka honey remained evident. The results reveal the capability of 5% (w/v) manuka honey to suppress the spread of soft-rot symptoms that were still active at 36 hpi.

Phylogenetic analysis of Usps

Phylogenetic analysis was conducted using the ML method to gain insights into the evolutionary relationship of Usp of *P. brasiliense* with other Enterobacteriales. The results show that the UspB and UspE of *P. brasiliense* were the most closely related to *P. carotovorum*, while UspA and UspG were most closely related to *P. polaris* (Figure 2). The orthologs of UspA and UspG belonged to three family groups: Pectobacteriaceae, Enterobacteriaceae, and Yersiniaceae.

The findings reveal that the UspA of *P. brasiliense* was closest to the *Pectobacterium* cluster and 98% closely related to *P. polaris* (WP_174878274.1). UspB was closest to Pectobacteriaceae and 100% closely related to *P. carotovorum* (WP_010281678.1). UspE showed a 99% close relation to *P. carotovorum* (WP_039531759.1). UspG was 99% closely related to *P. polaris* (WP_039480252.1).

Analysis of *usp* gene expression

The expressions of Usps increase as a cellular response to biotic and abiotic stresses. However, the results of RT-qPCR of *P. brasiliense* with 5% (w/v) manuka honey treatment showed that the expressions of all four *usp* genes were reduced to varying degrees (Figure 3).

The expressions of *uspA*, *uspB*, *uspE*, and *uspG* were downregulated by 5, 16, 10, and 62%, respectively. The largest decrease was observed in *uspG* and the smallest in *uspA*. This finding suggests that 5% (w/v) manuka honey weakened the cellular response of *P. brasiliense* to stress through decreased *usp* expression.

Discussion

In this study, a pathogenicity assay of *P. brasiliense* was carried out on a Chinese cabbage leaf, and the results revealed symptoms on the inoculation site. These symptoms presented the characteristics of soft-rot caused by *P. brasiliense* (Meng et al. 2017). Soft-rot symptoms appear as wet, soft, cream-colored decompressed tissue with odor. The spread of symptoms on Chinese cabbage leaves occurred vertically, as indicated by the lesion extending upward in the direction of the vein. This finding is similar to the research results of Lee et al. (2014), who showed that *P. brasiliense* inoculated on cabbage produced vertically spreading rot symptoms. Manuka honey can suppress soft-rot symptoms in Chinese cabbage. The lesions observed under the manuka honey treatment were smaller than those in the control (Figure 1). This decrease in virulence can be related to the downregulation of the four *usp* genes of *P. brasiliense* caused by exposure to manuka honey. Usps play a role in the survival of *Listeria monocytogenes* against oxidative stress from the host, indicating that Usps are an important virulence factor (Sibanda and Buys 2022). Likewise, Vollmer and Bark et al. (2018) stated that Usps are a global regulator associated with motility and biofilm formation.

Usps have been found in various organisms, including bacteria, metazoans, and plants (Chi et al. 2019). During their first discovery in *E. coli*, Usps were determined to play a role in various stress responses with several paralogs involved. In this research, four Usps, namely, UspA, UspB, UspE, and UspG, were studied. Several orthologs of Usps in *P. brasiliense*, diversely originate from Pectobacteriaceae, Yersiniaceae, and Enterobacteriaceae. Each bacterial species has a different number of paralogs. *E. coli* has 6 Usp (Luo et al. 2023), *Streptomyces coelicolor* has 12 (O'toole and Williams 2003), and *Xanthomonas campestris* has 1 (O'toole and Williams 2003). Three Usps have been found in *Brenneria rubrifaciens*, namely, UspA, UspB, and UspE. Meanwhile, *Yersinia ruckeri* has four Usps, namely, UspA, UspB, UspC, and UspE.

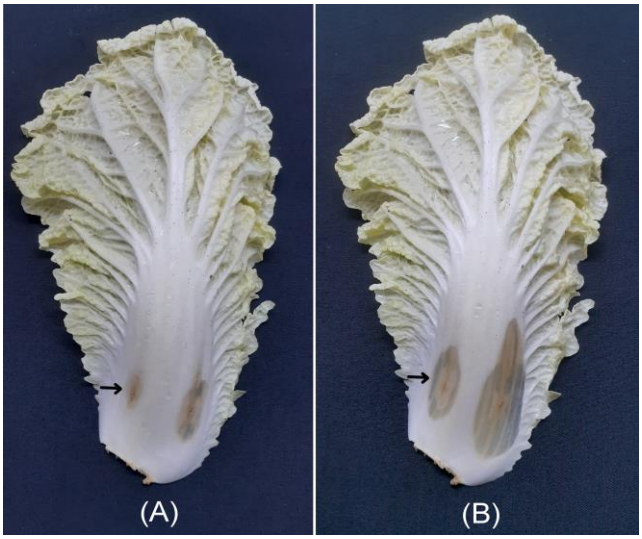

Figure 1. *Pectobacterium brasiliense* treated with 5% (w/v) manuka honey presented decreased maceration ability on Chinese cabbage at (A) 24 and (B) 36 hpi. The leaf was inoculated with 10 µL suspension of *P. brasiliense* (10^8 cells/mL) with 5% (w/v) manuka honey (indicated by arrow) and 10 µL *P. brasiliense* suspension without honey treatment

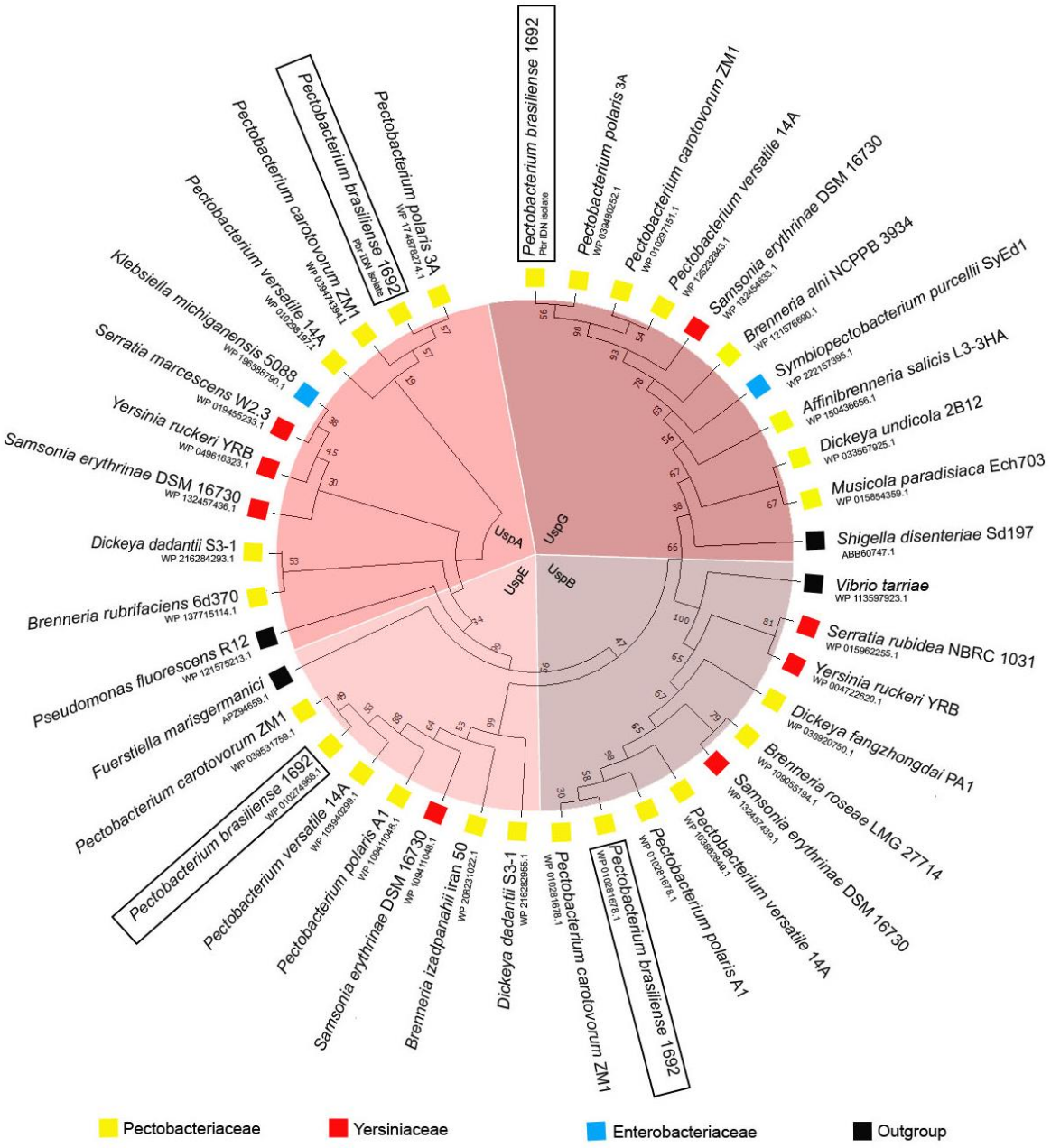

Figure 2. Phylogenetic tree based on the Usp of *Pectobacterium brasiliense* and its orthologs from Enterobacteriales constructed using MEGA 11 software. The branching pattern was generated via the ML method using the LG model based on BIC and AICc scores. Bootstrap support values are shown as percentages of 1,000 replications

Table 1. Primers sequences for *usp* expression analysis

Gene		Primer sequence (5' to 3')	Amplicon (bp)	References
recA	Forward	TGCGTTTATCGATGCTGAGC	134	Ava et al. 2022
	Reverse	AGCGCGTTAATGCATCACAG		
uspA	Forward	GGTTTCAATGGCAAGACCGTAC	140	This study
	Reverse	ACGCATTCTGGGTTTCTTCG		
uspB	Forward	GGCCAGCCAAGTAAACAAATCC	90	This study
	Reverse	GCAACGGCGAATAAACTCAGG		
uspE	Forward	AATGCGTCAGGGCGTTATTC	107	This study
	Reverse	TGTTGTGCCACACCACTTTG		
uspG	Forward	AGAATGAGGCGGTGGTGAAAG	101	This study
	Reverse	CGAGCCAAAACTGACGCTAC		

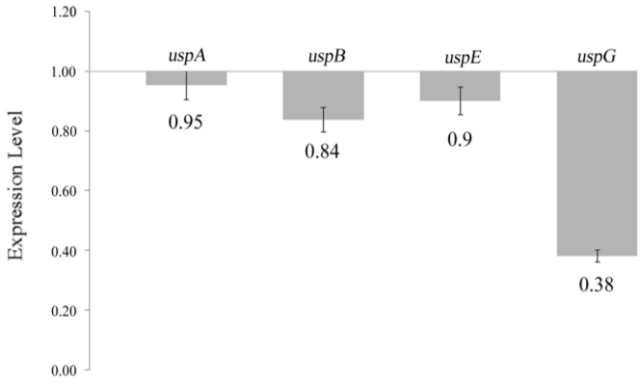

Figure 3. Manuka honey decreased the expressions of *uspA*, *uspB*, *uspE*, and *uspG* in *Pectobacterium brasiliense*. Description: 0% = no manuka honey; 5% = manuka honey treatment. Vertical bars represent the standard errors of three replications

The UspA of *P. brasiliense* is 99% closely related to *P. polaris* (WP_174878274.1), which also has four Usps. *K. pneumoniae* has UspA, UspB, UspC, UspE, UspG, and UspF, but only UspA is 94% closely related to UspA of *P. brasiliense*. The UspB of *P. brasiliense* is 100% closely related to the UspB of *P. carotovorum* (WP_010281678.1). *S. praecaptivus* has four paralogs, namely, UspA, UspB, UspE, and UspG, but only UspB shows a close relationship with UspB of *P. brasiliense* (82%). UspE is 99% closely related to *P. carotovorum* (WP_039531759.1). *B. rubrifaciens* has three Usps, namely, UspA, UspB, and UspE, but only UspE is closely related to UspE of *P. brasiliense* (95%). UspG is 99% closely related to UspG of *P. polaris* (WP_039480252.1). Orthologs of UspG from *B. rubrifaciens* and *Y. ruckeri* have not been found (Figure 2).

Jenkins et al. (2011) observed that manuka honey reduced UspA expression in *S. aureus*. This study analyzed the UspA expressions in *P. brasiliense* exposed to 5% (w/v) manuka honey. Previous research revealed that 5% (w/v) manuka honey effectively inhibited the growth of *P. brasiliense* (Ava et al. 2022). The 5% (w/v) manuka honey can inhibit the expressions of virulence genes related to extracellular enzyme production, motility, biofilms, and pellicles.

In this study, the effect of manuka honey on *usp* of *P. brasiliense* was investigated. All four *usp* of *P. brasiliense*, namely, *uspA*, *uspB*, *uspE*, and *uspG*, were affected by manuka honey (Figure 3). This result is consistent with that of Jenkins et al. (2011), who observed that manuka honey affected *uspA* expression. Interestingly, not only *uspA* of *P. brasiliense* but all four *usp* had decreased expressions. This finding shows the considerable effect of manuka honey on the decreased survival ability of *P. brasiliense*, as observed from the usp expression in adaption to stress.

The expressions of *uspA*, *uspB*, *uspE*, and *uspG* were downregulated by 5, 16, 10, and 62%, respectively. The most substantial decrease was observed in *uspG* and the smallest in *uspA*. The *uspA* increases cell survival during the stationary phase in *E. coli* (Nachin et al. 2005) and promotes the survival of *Salmonella typhimurium* in carbon or phosphorus starvation (Kroupitski et al. 2019). Meanwhile, the specific function of *uspA* of *P. brasiliense* is unknown. Nonetheless, the 5% decrease in expression implies that *uspA* played a role in the survival of *P. brasiliense* and was affected by manuka honey exposure.

Nachin et al. (2005) suggested that *uspA* plays a major role against oxidative stress. This assumption was proven by the remarkable increase in *uspA* expression when *E. coli* was challenged with hydrogen peroxide (H_2O_2). The *uspA* in *P. brasiliense* may also play a role in the survival against oxidative stress, as manuka honey contains H_2O_2, which plays an important role in antimicrobial activity (Majtan et al. 2014). Although H_2O_2 is the highest antibacterial compound in honey (30-50%), manuka honey contains less H_2O_2 than other kinds of honey (Johnston et al. 2018). The excellent antimicrobial activity of manuka honey is contributed by methylglyoxal (MGO) (Carter et al. 2016). MGO and H_2O_2 may serve as sources of oxidative stress that is unbearable to cells, as proven by the decreased expression of *uspA* of *P. brasiliense*.

According to Nachin et al. (2005), *uspG* also plays a role against oxidative stress but is less than that of *uspA*. This finding is in line with the results of this study, which reveal that *uspA* experienced a decrease in expression after exposure to manuka honey. Farewell et al. (1998) stated that *uspB* in *E. coli* is induced by several stresses, one of which is oxidative stress. The decrease in *uspB* expression in *P. brasiliense* may be due to the carbohydrate content (82.4%) and water, which create osmotic pressure (Chen et al. 2019). Meanwhile, *uspG* and *uspE* in *E. coli* play a role in intercellular aggregation (Nachin et al. 2005). The decreased expressions of *uspE* and *uspG* in *P. brasiliense* can be due to the MGO content of manuka honey. MGO can change the fimbriae and flagellar structure, thus inhibiting bacterial adherence (Nolan et al. 2019).

In conclusion, this study showed that four Usps of *P. brasiliense* are closely related to Pectobacteriaceae. In line with the disease suppression of *P. brasiliense* in Chinese cabbage due to manuka honey, the expressions of *uspA*, *uspB*, *uspE*, and *uspG* were downregulated after exposure to manuka honey. The largest decrease was observed in *uspG* and the smallest in *uspA*. The decreased virulence of *P. brasiliense* can be affected by the inability of the pathogen to survive in the host due to the decreased expression of *usp* genes as a global regulator. The study revealed that Usps play an important role in the pathogenicity of *P. brasiliense*, especially in adaption to environmental stresses. Thus, further experiments must be carried out to gain mechanistic insights into the functions of Usps.

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