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Evaluation of prebiotic utilisation and thermal tolerance of *Bacillus clausii* VTT and DP strains: Potential applications in the food industry

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Abstract:

Bacillus **species are promising probiotic bacteria because of their spore-forming ability, which enables them to survive in harsh environments, giving them an advantage over genera such as *Lactobacillus* and *Bifidobacterium*. While the effects of fibres such as fructo-oligosaccharide, inulin, and psyllium on *Lactobacillus* have been extensively studied, data on *Bacillus clausii* are scarce. This study investigated (1) the effects of fructo-oligosaccharide (FOS), inulin, and psyllium on the growth of *B. clausii* (VTT and DP) using OD_{600} measurements and (2) survival following heat treatment at 80°C for 10 min. The results showed that the *B. clausii* VTT strain exhibited enhanced growth with FOS and inulin at both 0.5 and 1%. In particular, 0.5% FOS resulted in the highest OD_{600} value (0.598±0.025) after 42 hours, significantly higher than the control (0.270±0.030). In contrast, psyllium inhibited VTT growth. The DP strain showed inconsistent responses to the prebiotics, and 1% inulin significantly reduced OD_{600} compared to the control. Both strains showed slight reductions in log colony-forming units (CFU)/ml after heat treatment, indicating high heat tolerance. These results demonstrate strain-specific differences in prebiotic responsiveness in *B. clausii* and identify FOS as an effective prebiotic for combination with *B. clausii* VTT, supporting the potential use of this combination in synbiotic formulations intended for high-temperature processing.**

Keywords: ***Bacillus clausii*****, growth response, prebiotic, synbiotic, thermal tolerance.**

Classification numbers: **3.4, 3.5**

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1. Introduction

According to the World Health Organisation (WHO), probiotics are defined as "live microorganisms that, when administered in adequate amounts, provide health benefits to the host". Among them*, Lactobacillus* and *Bifidobacterium* are the two most common genera in commercial preparations because of their ability to produce lactic acid through carbohydrate fermentation and contribute to maintaining gut microbiota balance [1]. However, these species are often sensitive to harsh conditions such as acidic pH, bile, and especially high temperatures during processing [2], thereby greatly reducing survival and bioactivity. Meanwhile, spore-forming bacteria such as *Bacillus clausii* have been shown to have outstanding heat resistance due to their endogenous spore-forming mechanisms, with a survival rate of up to 80% after heat treatment [3]. This suggests potential advantages for developing heat-resistant dietary supplements.

In addition to survival, the ability of probiotics to grow and maintain probiotic activity also depends on the availability of nutritional substrates, especially prebiotics. As defined by International Scientific Association for Probiotics and Prebiotics (ISAPP), a prebiotic is "a substrate that is selectively used by the host microorganisms that provides health benefits" [4], and when prebiotics are combined with probiotics, they may form the basis of a synbiotic formulation. Among popular prebiotics, FOS and inulin, two extensively studied fructans, have shown potent stimulatory effects on *Lactobacillus* and *Bifidobacterium*. However, data on the effects of prebiotics on *Bacillus* spp., especially *B. clausii*, are limited [5], making it difficult to design optimal synbiotic formulations.

Although *B. clausii* has been used in dietary supplements because of its natural antimicrobial properties, acid tolerance, and digestive-supporting properties, information on the interaction between this species and prebiotics is still limited [5]. Moreover, few studies have evaluated the heat resistance of individual *B. clausii* strains, an important characteristic for their potential application in functional products involving high-temperature processing. This gap not only limits the ability to select suitable prebiotics but also hinders the optimal exploitation of *B. clausii* in the food industry. Therefore, this study was conducted to (1) investigate the effects of three common prebiotics, FOS, inulin and psyllium, on the growth capacity of these strains through OD_{600} measurements and (2) evaluate the heat survival of two strains of *B. clausii* (VTT and DP) when treated at high temperature (80°C).

2. Materials and methods

2.1. Microbial strains and culture media

Two strains of *B. clausii* (VTT and DP) were isolated from faecal samples of healthy adults in Thai Binh province (before the administrative merger). The 16S rRNA gene sequences of these two strains (892 and 795 bp in length, respectively) were analysed using the BLASTn tool against the GenBank database. Both sequences showed 99.05-100% identity and 100% coverage with multiple reference strains of *B. clausii*, thereby confirming that the two isolated strains belonged to the species *B. clausii*. The isolates were stored on agar slants at 4°C for short-term use (up to two months) and -20°C for long-term storage. The bacteria were cultured in a standard Luria-Bertani (LB) medium consisting of 1% peptone, 0.5% yeast extract and 0.5% NaCl. The three prebiotics used in this study, namely NutraFlora® FOS Powder; Inulin Prebiotic Pure Powder, Organic; Psyllium Husk Powder, were commercial food-grade products from NOW Foods (USA) and purchased from local supermarkets in the Ba Dinh district (before the administrative merger) as commercial food-grade products. Based on the product labelling, no major additives likely to substantially influence bacterial growth were declared.

2.2. Research methods

2.2.1. Methods for determining cell density

OD_{600} is an optical turbidity measurement used to estimate microbial population density, based on the principle that bacterial cells are particles that scatter incident light (usually at a wavelength of 600 nm), thereby increasing turbidity, rather than absorbing light according to the Beer-Lambert law. Prior to inoculation, the initial inoculum was standardised to approximately 10^7 CFU/ml. The inoculation volume was adjusted based on OD_{600} measurements using a pre-established OD-CFU calibration curve for each strain, ensuring comparable initial bacterial concentrations across all experimental conditions. An aliquot of 200 µl of overnight culture was pipetted into each well of a 96-well plate to measure cell density based on optical turbidity (OD_{600}) [6-8]. OD_{600} values were blank-corrected using the corresponding uninoculated media for each prebiotic condition. All experiments were performed using three independent biological replicates for each time point and each prebiotic concentration.

2.2.2. Investigating the effects of prebiotics on the development of B. clausii

The selection of prebiotic concentrations was based on previously published studies. A study by M.Z. Wu, et al. (2022) [9] reported that *B. coagulans* BACO-17 has the ability to utilise FOS. M. Aulitto, et al. (2021) [10] and B.R. Tampangallo, et al. (2023) [11] reported that 1% inulin significantly enhanced the growth of *B. subtilis* BR610. Similarly, K.D. Shree, et al. (2017) [12] used 0.2, 0.3, and 0.4% psyllium husk for lactic acid bacteria. Based on these studies, with slight modifications for *B. clausii*, FOS and inulin were added separately to the LB medium at concentrations of 0, 0.5, and 1%, while psyllium was added at 0, 0.2, and 0.4% (summarised in Table 1). The cultures were incubated at 37°C with continuous shaking at 150 rpm. Samples were taken at seven time points (6-42 hours) to measure OD_{600}. Bacterial growth was estimated based on OD_{600} values.

Table 1. Prebiotic concentrations tested in the study.

Prebiotic	Tested concentration (%w/v)		
FOS	0	0.5	1
Inulin	0	0.5	1
Psyllium	0	0.2	0.4

2.2.3. Evaluation of heat survival of B. clausii under high-temperature conditions

To investigate the heat survival capacity, the two *B. clausii* strains, VTT and DP, were cultured in LB medium at 37°C for 22 hours. The culture was then transferred to a glass flask and heat-treated at 80°C for 10 minutes using a water bath to reduce vegetative cells and enrich heat-resistant bacterial populations. After heat treatment, the sample was serially diluted to 10^{-6}, and 100 µl aliquots from the last three dilution levels were spread onto LB agar plates and incubated at 37°C for 24 hours. Post-treatment survival was assessed by comparing the number of colonies after heat treatment with those at the initial time point, with counts expressed as log CFU/ml [13, 14]. The results allowed comparison of the heat survival capacities of the two strains, thereby evaluating their potential suitability for applications requiring thermotolerant probiotics in food production.

2.2.4. Statistical analysis

Data were analysed using GraphPad Prism 9.0. The effects of different prebiotic sources on bacterial growth were evaluated by one-way ANOVA with Tukey's post hoc test, with each time point analysed independently.

Accordingly, repeated-measures or time-course models were not applied. Heat survival between strains (expressed as log unit reduction after heat treatment) was compared using Welch's t-test due to unequal variances. Results are presented as mean±SD from three independent biological replicates.

3. Results

3.1. Impact of FOS, inulin and psyllium on the growth of B. clausii VTT strain

The overall results showed that *B. clausii* VTT responded differently to the three prebiotics tested (Fig. 1). Specifically, FOS and inulin were associated with markedly increased OD_{600} values compared to the control without prebiotic supplementation, while psyllium did not support enhanced growth at either tested concentration.

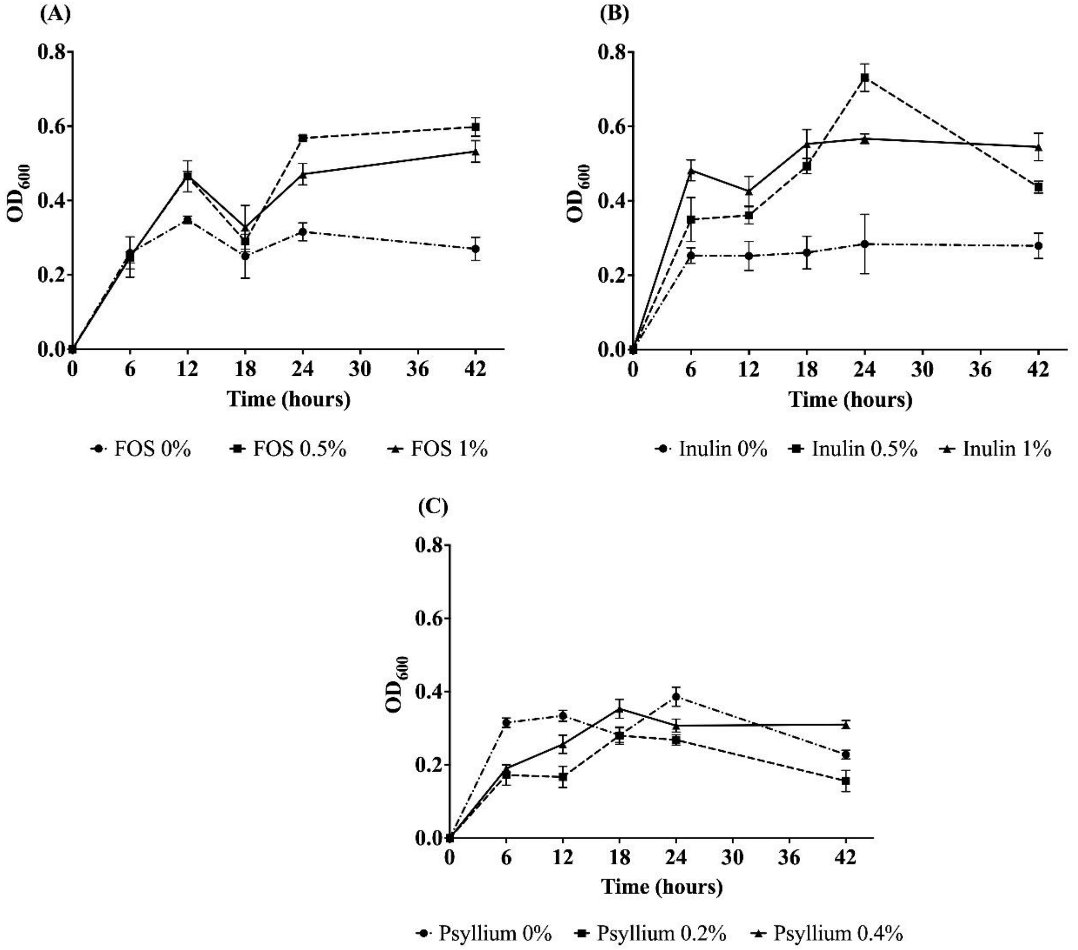

Fig. 1. Effects of the concentrations of (A) FOS, (B) inulin, and (C) psyllium fibre on the growth of *B. clausii* VTT strain over 42 hours. Data are presented as mean±SD (n=3).

With FOS (Fig. 1A), both the 0.5 and 1% concentrations supported significantly higher OD_{600} values over the entire culture period. The 1% FOS concentration was associated with a rapid increase in OD_{600} in the first 12 hours, but the 0.5% FOS concentration maintained a more sustained increase at later time points and reached the highest OD_{600} value at the 42-hour time point (0.598±0.025), outperforming the control (0.270±0.030, $p<0.0001$). However, no statistically significant difference was noted between the two FOS concentrations ($p=0.0664$). Similarly, inulin showed a positive OD_{600} response (Fig. 1B). Both 0.5 and 1% inulin supported higher growth than the control, peaking at approximately 24 hours, then decreasing at the 42-hour mark. There was no clear difference between the two inulin concentrations, suggesting that the observed OD_{600} response was not dose-dependent. In contrast, psyllium was associated with lower OD_{600} values compared to the control (Fig. 1C). Both 0.2 and 0.4% psyllium resulted in lower OD_{600} values than the control at most time points, especially after 18 hours. This suggests that, under the current culture conditions, psyllium was associated with less favourable growth-related OD_{600} responses for *B. clausii* VTT. Overall, FOS and inulin led to higher growth-related OD_{600} values for *B. clausii* VTT under the tested conditions, while psyllium showed less favourable OD_{600} responses.

3.2. Effects of FOS, inulin, and psyllium on the growth of B. clausii DP strain

For the *B. clausii* DP *strain*, the results of optical density monitoring (OD_{600}) over 42 hours showed different OD_{600} profiles in response to the three prebiotics (Fig. 2).

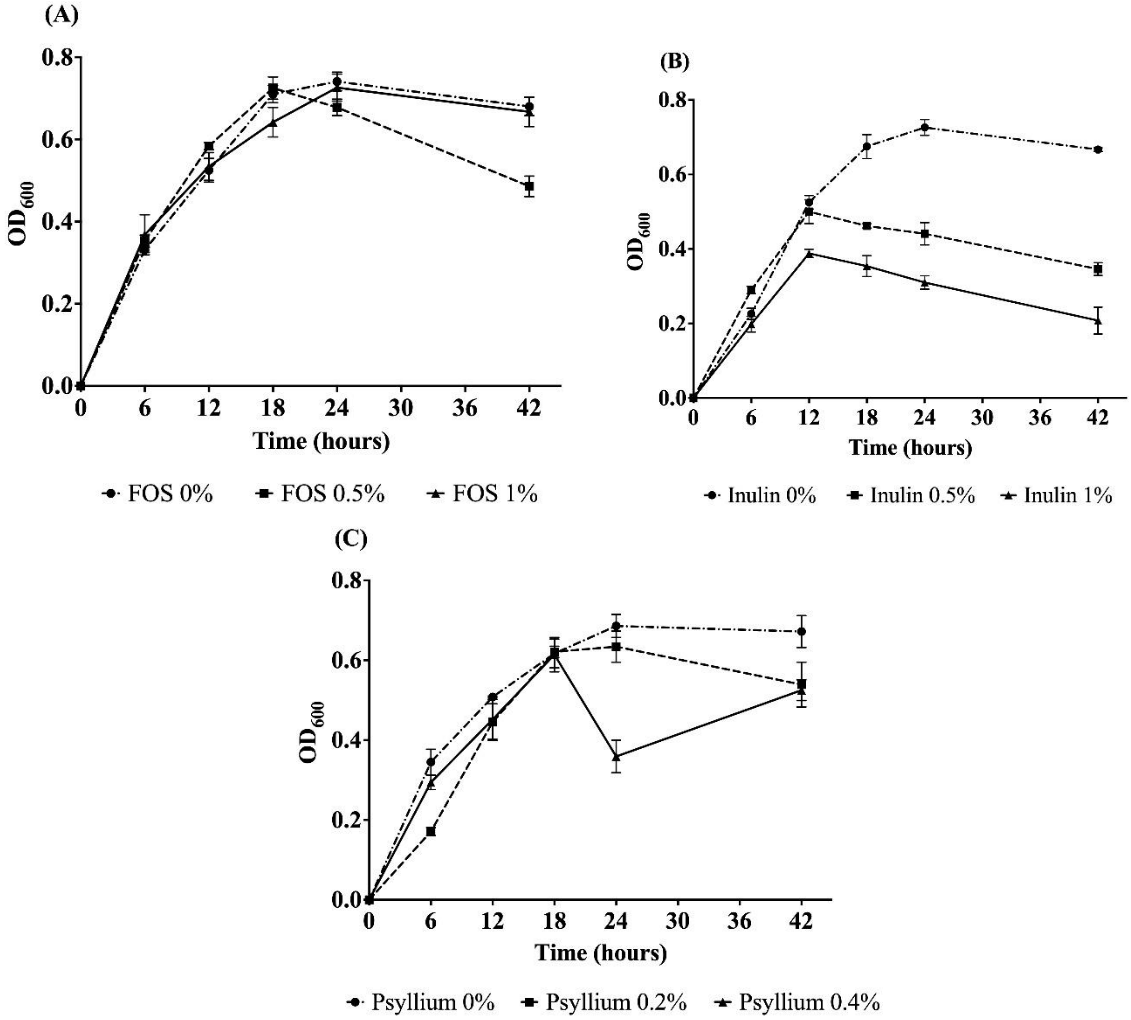

Fig. 2. Effects of the concentrations of (A) FOS, (B) inulin, and (C) psyllium fibre on the growth of the *B. clausii* DP strain over 42 hours.

With FOS supplementation (Fig. 2A), both the 0.5 and 1% concentrations were associated with increased OD_{600} values in the early stages, with OD_{600} peaking at 18 hours. However, after this point, the 0.5% FOS group showed a sharp decline in OD_{600}, while the 1% FOS group tended to be more stable over time, although remaining lower than the control at the 42-hour mark. Overall, FOS did not offer a clear OD_{600} advantage over the control medium, especially at later stages of the culture process. For inulin (Fig. 2B), both concentrations exhibited significantly lower OD_{600} values than the control group. The OD_{600} of the inulin-supplemented groups peaked early at 12 hours, then gradually decreased, suggesting a reduced OD_{600} response during prolonged incubation of *B. clausii* DP. In particular, 1% inulin was the most unfavourable condition, with OD_{600} decreasing continuously towards the end of the incubation period.

In contrast, psyllium (Fig. 2C) showed a relatively more favourable OD_{600} profile. Neither the 0.2 nor the 0.4% concentration enhanced bacterial growth compared with the control during the 12-24-hour period, although the OD_{600} values increased steadily after 18 hours of incubation. At 42 hours, the OD_{600} of the psyllium groups remained comparable to the control, indicating that psyllium did not result in a marked reduction in OD_{600} values compared to the control at later time points.

Overall, the results showed that *B. clausii* DP responded inconsistently to prebiotics: inulin exhibited a pronounced negative effect, FOS was only effective during the early stages, whereas psyllium exhibited a more stable OD_{600} profile over time compared to FOS and inulin.

3.3. Comparison of growth-related OD_{600} responses at 42 hours

For *B. clausii* VTT, both FOS and inulin were associated with significantly higher OD_{600} values compared to the control, indicating enhanced growth-related OD_{600} responses (Figs. 3A, 3C). In particular, the 0.5% FOS resulted in the highest value, which was only slightly higher than that observed with 0.5% FOS, suggesting that FOS concentration influenced the magnitude of the OD_{600} response, although the difference between concentrations was limited (Fig. 3A). Inulin also showed a positive but less pronounced OD_{600} response compared with FOS (Fig. 3C). In contrast, psyllium exhibited a pronounced negative effect. Both 0.2 and 0.4% psyllium resulted in lower OD_{600} values compared with the control, indicating an unfavourable profile for this strain under the tested conditions (Fig. 3E).

The *B. clausii* DP strain showed low growth levels under all tested prebiotic conditions (Figs. 3B, 3D, 3F). Specifically, the OD_{600} value in the prebiotic-supplemented conditions was lower than that of the control, especially with inulin, which showed the lowest value. These results suggest that, unlike the VTT strain, *B.clausii* DP exhibited consistently lower OD_{600} values in the presence of prebiotic supplementation compared with the control.

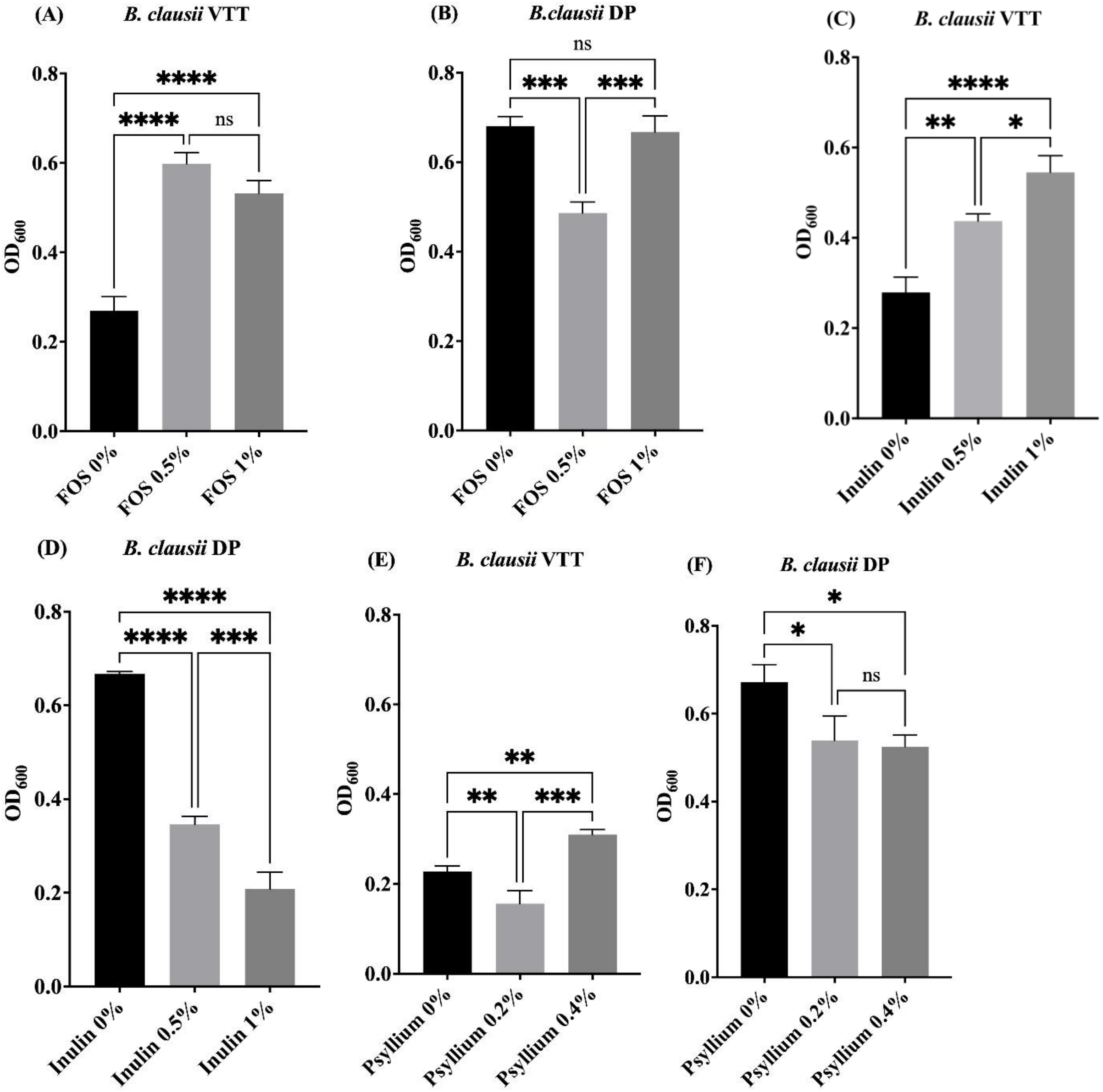

Fig. 3. OD$_{600}$ **values of *B. clausii* VTT and DP after 42 hours of culture in media supplemented with FOS (A, B), inulin (C, D), and psyllium (E, F).** Significance level: ns=not significant, p<0.05 (*), p<0.01 (**), p<0.001 (***), p<0.0001 (****).

3.4. Survival of B. clausii DP and VTT after heat treatment

The survival of the two *B. clausii* strains (DP and VTT) was evaluated after heat treatment at 80°C for 10 min. The number of surviving cells was quantified before and after heat treatment and expressed as log CFU/ml, with three biological replicates for each condition.

Before heat treatment, the DP strain had a higher cell density than VTT (9.235±0.377 compared to 7.990±0.294 log CFU/ml), possibly reflecting differences in growth characteristics. After treatment, both strains showed slight reductions: DP and VTT decreased to 8.347±0.576 and 7.516±0.495 log CFU/ml, corresponding to survival rates of 90.39 and 94.06%, respectively, and representing decreases of approximately 0.889 and 0.474 log units.

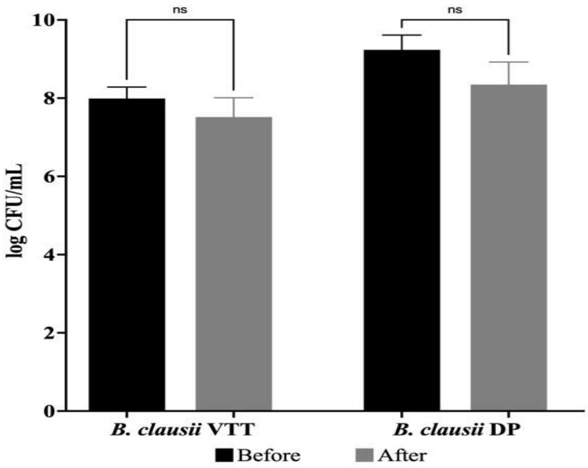

Fig. 4. Survival of *B. clausii* VTT and DP strains before and after heat treatment (80°C for 10 min). Significance level: ns=not significant.

Statistical analysis by Welch's t-test showed that the difference between the reductions observed in the two strains was not statistically significant (Fig. 4). This suggests that, despite the difference in initial density, both strains were similarly capable of surviving heat treatment, which may be related to intrinsic heat tolerance.

Overall, the results showed that both *B. clausii* DP and VTT had high post-treatment survival, indicating that both strains exhibited measurable survival following the applied heat treatment.

4. Discussion

4.1. Strain specificity in probiotic-prebiotic interactions

The *B. clausii* VTT *strain* responded positively to FOS and inulin, especially at a concentration of 0.5%, with a marked increase in OD_{600} values compared to the control group. Meanwhile, *the B. clausii* DP strain did not exhibit steady growth with prebiotic supplementation, and even 1% inulin significantly reduced growth, as reflected in a continuous decrease in OD_{600} values towards the end of the incubation period. These results clearly demonstrate strain-specific responses to prebiotics. This phenomenon was also reported by M.Z. Wu, et al. (2022) [9], who showed that different *Bacillus* strains utilised FOS, GOS, or pectin differently depending on their enzymatic profiles. Similarly, T. Sukul, et al. (2023) [15] noted that FOS can promote growth and enhance the production of extracellular enzymes.

The inhibitory effect of psyllium may be attributed to its high viscosity and to metabolic interference associated with its arabinoxylan structure. J. Jalanka, et al. (2019) [16] also reported similar effects on the culture microenvironment. Furthermore, the differences between the two strains may be due to variations in their enzyme systems. Many *Bacillus* strains, such as *B. velezensis* [17] or *B. coagulans* MA-13 [10], possess enzymes such as fructofuranosidase and levansucrase, which help hydrolyse prebiotics.

The poor DP response may suggest a deficiency in the relevant carbohydrate-utilising enzymes, which should be verified by gene sequencing in the future.

Ultimately, these findings emphasise the importance of selecting appropriate probiotic-prebiotic pairs. While FOS appears to be a promising prebiotic for *B. clausii* VTT, the optimal prebiotic for DP requires further investigation. Additionally, both strains showed high survival after heat treatment, supporting their potential relevance for applications involving thermal processing.

In the present study, each time point was obtained from independently prepared culture samples rather than repeated measurements from the same culture vessel. Therefore, each time point was analysed independently to compare OD_{600} responses under different prebiotic conditions. However, analysing multiple time points separately may still increase the possibility of multiple testing effects and may not fully capture overall temporal growth dynamics. Future studies using more advanced longitudinal experimental designs may provide a more integrated evaluation of bacterial growth behaviour over time.

4.2. Heat survival and potential industrial applications

After heat treatment at 80°C for 10 min, both *B. clausii* VTT and DP strains retained high survival rates (94.06 and 90.39%, respectively) (Table 2), indicating strong heat tolerance under the applied laboratory conditions. This level of survival highlights their potential suitability for applications involving thermal exposure within the scope of the current method.

Table 2. Comparison of post-heat-treatment survival among *B. clausii* strains.

Strain	Treatment conditions (°C/min)	Survival rate	Reference
B. clausii VTT	80/10	94.06%	This study
B. clausii DP	80/10	90.39%	This study
B. clausii OHRC1	90/30	96.31%	R. Chelliah, et al. (2024) [18]
B. clausii ANA39	80/20	About 80%	T. My, et al. (2022) [3]
Shouchella clausii BCLA	90/3	31.7% (16.6×10^8 CFU/ml)	N.H.M. Duc, et al. (2024) [19]
Shouchella clausii BCLA	75/3	81.1% (42.4×10^8 CFU/ml)	N.H.M. Duc, et al. (2024) [19]
B. clausii CSI08	90/3	43.8% (3.01×10^9 CFU/ml)	E. Khokhlova, et al. (2023) [20]

The study by T. My, et al. (2022) [3] reported that *B. clausii* ANA39 strain achieved a survival rate of about 80% after treatment at 80°C for 20 min, a treatment duration twice that used in the current study.

Notably, *B. clausii* OHRC1 exhibited a survival rate of up to 96.31% at 90°C for 30 min [18]. In contrast, E. Khokhlova, et al. (2023) [20] reported a considerably lower survival rate for *B. clausii* CSI08 (43.8%) under 90°C for 3 mins, indicating strain-dependent differences in spore resistance even under similar treatment temperatures. Meanwhile, N.H.M. Duc, et al. (2024) [19] reported that *Shouchella clausii* BCLA had a survival rate of 31.7% at 90°C, but reached 81.1% at 75°C, suggesting favourable heat tolerance within the range of 75-80°C.

Overall, these results confirm that *B. clausii* is a highly heat-resistant spore-forming bacterium that may be advantageous for probiotic applications that require heat treatment in industrial processes, where many *Lactobacillus* and *Bifidobacterium* strains show reduced survival. While the laboratory conditions (80°C) indicate good survival, further studies simulating specific industrial processes, such as spray drying or pasteurisation, are needed to validate their suitability. Additionally, it should be noted that the method of CFU enumeration after heat treatment does not directly distinguish between spores and surviving vegetative cells.

Therefore, to draw firm conclusions about sporulation efficacy, additional analyses such as spore staining or ethanol treatment would be required in further studies.

5. Conclusions

This study demonstrates clear strain-specific differences in the growth-related OD_{600} responses and heat treatment survival of *B. clausii* VTT and DP. Under the tested conditions, FOS and inulin were associated with enhanced growth-related OD_{600} responses in the VTT strain, whereas the DP strain exhibited less pronounced and more variable responses across the tested prebiotics.

Despite differences in growth-related OD_{600} responses, both strains demonstrated high survival after heat treatment at 80°C for 10 min, highlighting their strong heat tolerance under the tested conditions. Within the scope of the current approach, the observed survival reflects overall heat resistance, while further analyses would help clarify the contribution of heat-resistant forms.

Overall, the findings suggest that prebiotic effects on *B. clausii* are highly strain-dependent and that careful selection of fibre type is required when evaluating synbiotic combinations. Further studies incorporating complementary growth and sporulation-specific analyses are needed to better assess the functional relevance of these observations.

CRediT author statement

Phuong Thao Nguyen: Investigation, Formal analysis, Data curation, Visualisation, Writing original draft; Hai Ngan Doan, Thanh Tra Nguyen, Thi Dung Pham: Investigation; Thi Tuyet Nhung Nguyen: Conceptualisation, Methodology, Supervision, Project administration, Resources, Validation, Funding acquisition, Writing - Review & Editing.

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COMPETING INTERESTS

The authors declare that there is no conflict of interest regarding the publication of this article.

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