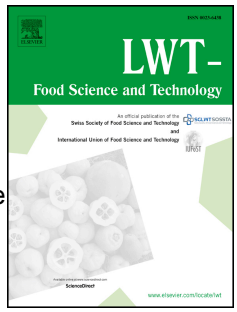

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Author contributions

Zhenbo Xu: Funding acquisition, Validation; **Ziqi Liu:** Writing-Original draft preparation, Data curation; **Thanapop Soteyome**: Methodology; Supervision; **Yang Deng**: Methodology; Supervision; **Gamini Seneviratne**: Validation; **Janette M. Harro & Birthe V. Kjellerup**: Writing-review & editing; **Junyan Liu**: Conceptualization, Validation, Writing-review & editing.

A novel procedure in combination of genomic sequencing, flow cytometry and routine culturing for confirmation of beer spoilage caused by *Pediococcus damnosus* in viable but nonculturable state

Zhenbo Xu[1,2,3,4,5,6], Ziqi Liu[1], Thanapop Soteyome[4], Yang Deng[7], Ling Chen[1], Gamini Seneviratne[6], Junyan Liu[8*], Janette M. Harro[9] and Birthe V. Kjellerup[8]

[1] School of Food Science and Engineering, Guangdong Province Key Laboratory for Green Processing of Natural Products and Product Safety, South China University of Technology, Guangzhou 510640, China

[2] State Key Laboratory of Applied Microbiology China Southern; Insititue of Microbiology, Guangdong Academy of Sciences 510070, China

[3] Department of Laboratory Medicine, the Second Affiliated Hospital of Shantou University Medical College, Shantou, China

[4] Home Economics Technology, Rajamangala University of Technology Phra Nakhon, Bangkok, Thailand

[5] Overseas Expertise Introduction Center for Discipline Innovation of Food Nutrition and Human Health (111 Center), Guangzhou 510640, China

[6] National Institute of Fundamental Studies, Kandy, Sri Lanka

[7] College of Food Science and Engineering, Qingdao Agricultural University, Qingdao 266109, China

[8] Department of Civil and Environmental Engineering, University of Maryland, College Park, MD 20742, USA

[9] Department of Microbial Pathogenesis, School of Dentistry, University of Maryland, Baltimore, MD 21201, USA

***Corresponding author:**

Junyan Liu, Ph.D.

Mailing address: Department of Civil and Environmental Engineering, University of Maryland, 0162 Glenn L Martin Hall, College Park, MD 20742, USA

E-mail address: yaner0722@hotmail.com

Abstract

Spoilage bacteria had been shown to form viable but nonculturable (VBNC) state maintaining food spoilage capability. In this study, a novel procedure was used to confirm a beer spoilage case caused by a *Pediococcus damnosus* strain in the VBNC state. Firstly, flow cytometry, routine culturing and PMA-PCR methods were used to identify approximately 10^3 cells/ml VBNC cells in the spoiled beer sample based on the difference between CFU and viable cell numbers. Secondly, genomic sequencing showed all acquired scaffolds were identical to the genome of *P. damnosus* with no existence of other species or isolates. In addition, VBNC cells were obtained in both simulation conditions, including beer low temperature storage and subculturing. MRS agar supplemented with catalase was found to resuscitate VBNC cells. Normal, VBNC and resuscitated cells showed similar level of beer spoilage capability. As concluded, a novel procedure, in combination of genomic sequencing, flow cytometry and routine culturing was used to confirm VBNC cells in spoiled beer sample, providing direct evidence on the beer spoilage caused by VBNC *P. damnosus* cells, and will aid in further study on VBNC state in food industry so that more evidence on food safety problem caused by VBNC microbes will be shown.

Keywords: beer spoilage; *Pediococcus damnosus*; viable but nonculturable state (VBNC); resuscitation; flow cytometry

1. Introduction

Bacterial cells in viable but nonculturable (VBNC) state has posed a major issue for food quality as VBNC cells yields false negative results during microbiological identification by culturing. The VBNC state has been considered to be a survival strategy of over 80 non-sporeforming bacterial species in response to environmental stress conditions (Yamamoto, 2000). Microorganisms in the VBNC state do not grow in routine culture media, but are still metabolically active and usually capable of resuming growth when certain stress conditions are relieved (Oliver, 2010). Problems posed by VBNC state bacteria are mainly due to the nonculturability in conventional laboratory media, leading to the difficulty in detection, along with the active metabolism (Sachidanandham & Gin, 2009). Interestingly, *in vitro* developed fungal-bacterial biofilms and their exudates have been shown to resuscitate VBNC, which might open an avenue to culture VBNC in the laboratory setting (Buddhika & Seneviratne, 2019). The VBNC state of pathogenic bacteria including *Vibrio cholerae* and enteropathogenic *Escherichia coli* has been intensively studied and suggested to be the recessive cause of some human diseases (Colwell et al., 1996; Makino et al., 2000). However, problems caused by VBNC state bacteria in food industry have been neglected. Food and its surrounding environment are a complex and could be factors inducing the VBNC state of foodborne bacteria (Mougin et al., 2019; Cao et al., 2019; Chen et al., 2019). *E. coli* O157:H7 and *Salmonella typhimurium* have been demonstrated to enter into the VBNC state in refrigerated pasteurized grapefruit juice

(Nicolo et al., 2011). VBNC state foodborne pathogens including *Salmonella enterica*, have been identified to be responsible for foodborne outbreaks (Asakura et al., 2002). Furthermore, acetic acid and lactic acid bacteria have been reported to enter into the VBNC state in wine and beer, and VBNC lactic acid bacterial cells have even caused beer spoilage cases (Liu et al., 2018a). Thus, the VBNC state of foodborne pathogens and spoilage bacteria has been a challenge in food safety. Up to date, a number of spoilage bacteria have been shown to be capable of forming VBNC state, and cells in VBNC state are viable and maintain food spoilage capability. However, a critical concern remains as it has never been confirmed the VBNC cells cause such food spoilage. Thus, culturing based microbial detection method is still golden standard in food industry as no direct evidence showing the existence of VBNC cells in food products and future cause food spoilage or poisoning case.

In the current study, we aimed at using a novel procedure, in combination of genomic sequencing, flow cytometry and routine culturing, to confirm a beer spoilage case is caused by *Pediococcus damnosus* in the VBNC state.

2. Materials and methods

2.1 Bacterial strain and culture condition

A spoiled beer sample (lager beer, pH 4.5, ethanol ≥ 3.6% v/v, bitterness units 7, stored at 4°C for 3 months after manufacturing with an expiration time of 12 months) with turbidity and undesired smell was acquired from a local brewery in 2014. Following culturable and viable cell numbers assessment, *16S rRNA* sequencing, and

genomic sequencing, one *P. damnosus* strain, designated BM-PD14610, was harvested from the spoiled beer sample. During the identification process, the spoiled beer sample was maintained at 4°C. The harvested cells were maintained as glycerol stock at -80°C. A small amount of glycerol stock was spread onto MRS agar (Oxoid, UK) and incubated at 26°C for 3-7 days. A single colony was transferred to 1 mL of MRS broth (Oxoid, UK) and incubated anaerobically at 26°C, 200 rpm for 24 h to obtain logarithmic growing cells prior to further experiments.

2.2 Determination of VBNC cells

The existence of VBNC cells was determined by cell viability and culturability test. Cell viability test was performed using a Live/Dead BacLight bacterial viability kit (Molecular Probes, USA) combined with flow cytometry. Two fluorescent dyes propidium iodide (PI) and SYTO 9 were applied to indicate permeabilized and total cells, respectively. In brief, the fluorescent dyes PI and SYTO 9 were mixed in a 2:1 ratio. Washed cell samples were stained with dye mixture in the dark for 20 min. Logarithmic growing cells and heat-killed cells were served as positive controls for viable and permeabilized cells, respectively. Thus, viable and permeabilized cell numbers could be assessed by NovoCyte flow cytometer with NovoExpress 1.2.5 software (ACEA Biosciences, Inc.). With SYTO 9 (FITC) as x-axis and PI (PIPE-Texas Red) as y-axis, the cells fall into the fourth quadrant are identified to be viable cells. Culturable cells enumeration was carried out by routine MRS agar plate counting. For regular identification, 100 μL of culture were plated on MRS agar plate followed by 48

h incubation at 26°C. For enrichment, 10 mL of sample were enriched by centrifugation at 1,000 ×g for 10 min and resuspended in 100 μL of distilled water prior to MRS agar plating. The difference between viable and culturable cell numbers was designated to be VBNC cell number. When culturable cell number reached 0 (no colony appears when plating 100 μL culture on plate), all the viable cells entered into the VBNC state.

2.3 16S rRNA and Genome sequencing

To identify the bacterial species causing the spoilage of the beer sample, both culturable and viable cells in 500 mL of the spoiled beer sample were enriched by centrifugation at 1,000 ×g for 10 min and adapted to DNA isolation using a Bacterial genomic DNA extraction kit (Dongsheng Biotech Co., Ltd, China) according to the instruction. The genomic DNA was used as a template to amplify *Lactobacillus 16S rRNA* gene (F: AGAGTTTGATCCTGGCTCAG, R: CTACGGCTACCTTGTTACGA) and *Pediococcus 16S rRNA* gene (F: CTACGGGAGGCAGCAAG, R: ATTACCGCGGCTGCTGG). The PCR product was purified and adapted to Sanger sequencing (IGE Biotech LTD, China). The sequence was aligned to the currently available sequences in GenBank using BLASTn.

To identify if the *P. damnosus* cells in the spoiled beer sample are from the same strain, the genomic DNA of *P. damnosus* cells from the spoiled beer sample was also sequenced by the Illumina HiSeq 2500 platform and paired-end libraries. The read qualities were examined and filtered by FastQC v.0.10.1. The filtered reads were assembled *de novo* into scaffolds through Velvet software v1.2.08 (Zerbino & Birney

2008).

2.4 Confirmation of VBNC state

To confirm the existence of VBNC state, nonculturable cells were further adapted to propidium monoazide (PMA)-PCR assay (Liu et al., 2017a). As PMA penetrates only into permeabilized bacterial cells with compromised membrane integrity but not into live cells with intact cell membranes, PMA treatment to cultures with both viable and dead cells result in selective removal of DNA from dead cells. The positive amplification of PMA treated nonculturable cells indicates the existence of viable cells (i.e. VBNC cells). PMA was added to nonculturable cells and the mixed samples were incubated on ice in dark for 10 min. Cooled samples were exposed to halogen light (with a distance of 15 cm) for 5 min for covalent binding of PMA to DNA. PMA binding DNA was extracted using a Bacterial genomic DNA extraction kit (Dongsheng Biotech Co., Ltd, China) according to the instruction and used as a template to amplify *P. damnosus 16S rRNA* gene (F: CTACGGGAGGCAGCAAG, R: ATTACCGCGGCTGCTGG). PCR were conducted following the cycling program: an initial heating at 94°C for 3 min, followed by 30 cycles of 94°C for 50 s, 51°C for 50 s, and 72°C for 1 min, with a final 10 min extension at 72°C. PMA-PCR were conducted in triplicate to ensure reproducibility.

2.5 Low temperature storage system

The low-temperature storage system was set up to mimic the beer storage process, a potential stress condition to *P. damnosus*. Approximately 10^7 logarithmic growing *P.*

155 *damnosus* cells were inoculated and anaerobically cultured at 26°C in 10 mL of
156 degassed and autoclaved commercial beer. The logarithmic growing cells were
157 harvested at 4°C (centrifugation at 2800 ×g for 15 min) and washed twice with
158 phosphate buffer (PBS). Then the washed cells were filtered and resuspended in 10 mL
159 of degassed and autoclaved beer at a final density of 10^7 cells/mL and maintained at
160 0°C without shaking. VBNC state determination was performed every 7 days.

161

162 2.6 Beer subculturing system

163 The beer subculturing system was performed to mimic the beer processing as
164 previously described (Deng et al., 2015). The samples preparation was performed as
165 described in the low-temperature storage system set up. The cells were filtered and
166 resuspended in 10 mL of degassed and autoclaved beer at a final density of 10^7 cells/mL
167 and anaerobically cultured at 26°C (1^{st} generation). After 7 days incubation, cells from
168 10 ml 1^{st} generation were filtered and reinoculated in fresh degassed and autoclaved
169 beer at 26°C (2^{nd} generation). The interval of each subculture and VBNC state
170 determination were 7 days.

171 2.7 Resuscitation strategies

172 The VBNC cells (approximately 10^5 cells/mL) acquired from the low temperature
173 and beer subculturing system were subsequently used for resuscitation. Temperature
174 upshift and chemical addition were applied for VBNC cells resuscitation, respectively.

Temperature upshift was performed by initially incubating the VBNC cells in MRS broth at 10°C for 1 h and gradually increasing the temperature by 5°C every 1 h until reaching 35°C. Cell culturability was determined every hour. MRS broth and agar plates with the addition of 10 μL Tween-20, 10 μL tween-80, 0.05 g vitamin C, 0.05 g vitamin B2, and 800 U/plate catalase (Sigma-Aldrich, USA) were used, respectively, in chemical addition system (Liu et al., 2018ab). Different temperatures were also used for the chemical addition system in MRS broth.

2.8 Beer contamination test

Approximately 10^6 of logarithmic growing, VBNC, and resuscitated *P. damnosus* cells were inoculated into 10 mL of degassed and autoclaved beer samples at room temperature, respectively. Uninoculated beer sample was used as negative control. The beer turbidity was visually observed every 7 days. Organic acids and diacetyl concentrations were determined after 30 days by reversed-phase high performance liquid chromatography (RP-HPLC) and head space gas chromatography, respectively, and quantified by the external standard method (Liu et al., 2017bcd).

2.9 Statistical analysis

Data are presented as mean ± standard deviation of three independent biological replicates. Statistical comparisons were performed by one-way analysis of variance followed by Tukey's comparison test (XLstat software). A p-value < 0.05 was considered to be significant.

3. Results

3.1 *P. damnosus* cells in VBNC state cause beer spoilage

One spoiled beer sample which had been stored at 4°C for 3 months, was subjected to routine MRS agar culturing detection to identify contaminating source, as most of the beer spoilage cases had been reported to be caused by lactic acid bacteria (LAB). No colony appeared on MRS agar plate with 1× beer sample, suggesting the existence of VBNC state bacteria. However, less than 10 colonies appeared in the enriched beer sample, indicating the presence of small amount of culturable cells. Thus, viable cell number was assessed to determine the existence of VBNC cells. The difference between culturable (less than 10 cells/mL) and viable cell number was approximately 5×10^2 cells/mL, showing the presence of VBNC cells in the spoiled beer sample. Thus, the bacterial cells from the spoiled beer sample were collected and subjected to further identification. According to our previous studies, VBNC state *Lactobacillus harbinensis* had been determined to cause beer spoilage case (Liu et al., 2018a), and some other Lactobacillus spp. strains including *L. brevis* (Liu et al., 2018b), *L. casei* (Liu et al., 2017c), *L. acetotolerans* (Deng et al., 2015), *L. plantarum* (Liu et al., 2017d), and *L. lindneri* (Liu et al., 2017b) were also capable of causing beer spoilage and entering into the VBNC state. *Lactobacillus 16S rRNA* gene amplification was performed on the nonculturable cells from the spoiled beer sample. Unfortunately, the bacterial cells were not *Lactobacillus* spp. Besides *Lactobacillus* spp., *Pediococcus* spp. are also one of the most common beer spoilage bacteria. *Pediococcus* spp. are generally

considered to be the most undesirable contaminant (Xu et al., 2020), with *P. damnosus* responsible for 90% of *Pediococcus*-induced beer spoilage (Kaiala et al., 2018; Behr, Gessler, Schmid, Zehe, & Vogel, 2016). Thus, *Pediococcus 16S rRNA* gene was amplified and sequenced to determine the nonculturable cells to be *P. damnosus*. In addition, the presence of VBNC cells was confirmed by PMA-PCR, verifying the beer spoilage case was caused by the co-existence of both culturable and VBNC state *P. damnosus* cells. Furthermore, genome sequencing was performed to identify the *P. damnosus* cells. Assembly and alignment results demonstrating the culturable and VBNC cells in the spoilage sample belong to the same *P. damnosus* strain (designated BM-PD14610, GenBank accession number: LTEA00000000).

3.2 VBNC state induced by stress conditions

To simulate the beer storage and processing conditions, low temperature storage and beer subculturing systems which had previously been shown to induce the VBNC state of several beer spoilage *Lactobacilli* (Deng et al., 2015; Liu et al., 2017bcd; Liu et al., 2018ab) were tested on *P. damnosus* strain BM-PD14610 strain, respectively. Both strategies were capable of inducing *P. damnosus* cells entry into the VBNC state (Fig. 1). At low temperature storage, viable cell number was significantly higher than culturable cell number since day 21 (p value <0.05), with 81.4% of the cells entered into the VBNC state. All the viable cells (4.4×10^4 cells/mL) entered into the VBNC state since day 133. In beer subculturing experiments, the ratio of VBNC cells was 86.6% after the 1st generation (day 7) and reached 100% after the 17th generation (day 119)

with a cell number of 2.3 x 10^5 cells/mL. The shorter time for entering into the VBNC state and the higher amount of VBNC cells observed in the beer subculturing system by comparison to the low temperature storage data, indicated the cold stress was not an essential factor for the VBNC state formation of *P. damnosus* strain. Considering cold stress is a common induction condition and capable of inducing multiple *Lactobacillus* species into the VBNC state (Deng et al., 2015; Liu et al., 2017bcd; Liu et al., 2018ab), it might be a facilitating factor for the VBNC state formation of *P. damnosus* strain. In addition, acetic acid concentration had identified to be the greatest effect on the formation of VBNC state of *P. acidilactici*, followed by nutritional conditions and salt concentration (Li et al., 2020). Thus, although belonging to the same species, *P. damnosus* and *P. acidilactici* entered into the VBNC state in different conditions.

3.3 Resuscitation of VBNC cells

To collect more culturable cells of *P. damnosus* strain BM-PD14610 for further study, the harvested VBNC cells were subjected to resuscitation. VBNC state has been considered a survival strategy of bacterial cells under stress conditions and partial portion of the VBNC cells was capable of resuscitating when certain stresses alleviated (Oliver, 2010). Considering the cold, oligotrophic and oxidative stress conditions that might be suffered by the *P. damnosus* cells during beer processing and cryopreservation, temperature upshift and chemical addition were tested to relief stress and resuscitate the VBNC cells. However, temperature upshift, addition of Tween-20, tween-80, vitamin C, or vitamin B2 did not enable VBNC cells resuscitation, indicating cold and

oligotrophic stress were not the key conditions for the VBNC state formation of *P. damnosus* strain. With the addition of catalase, which had been reported to relieve oxidative stress and promote the recovery of nonculturable cells (Kong et al., 2014; Jallouli, Zouari, & Jaoua, 2010), culturable cells of *P. damnosus* strain were obtained within 3 days. High concentration of hop bitter compounds in beer had been reported to exert antibacterial effect and transmembrane redox reactions, causing intracellular oxidative damage (Behr & Vogel, 2010). The VBNC *P. damnosus* cells might be stressed and sensitive to oxidative stress and induce VBNC state as an adaptation to resist hop compounds encountered in beer. Thus, the antioxidant capacity of catalase may alleviate the stress posed to *P. damnosus* cells. Coupled with the induction results, oxidative stress posed by beer might play important role for *P. damnosus* to enter into the VBNC state.

3.4 Beer contamination by VBNC cells

According to the beer contamination test, logarithmic growing, VBNC, and resuscitated *P. damnosus* cells caused visually beer turbidity and undesirable smell and flavor in 7 days. Although the organic acids and diacetyl produced by VBNC cells were lower than those produced by logarithmic growing and resuscitated cells, the VBNC cells were capable of causing unbalanced acid and diacetyl contents in beer (Table 1). The results verified VBNC *P. damnosus* cells were the cause of beer spoilage case. Thus, besides *Lactobacillus* spp. (Deng et al., 2015; Liu et al., 2017bcd; Liu et al., 2018ab), the detection and control of VBNC state *Pediococcus* spp. strains are also of importance

279 in beer industry.

280 4. Discussion

281 Based on years of study in the field of VBNC state, an important concern about
282 the food safety issues caused by bacteria in VBNC state has been raised. The issue
283 caused by VBNC state in food industry is common, not only for spoilage microbes, but
284 also for pathogenic microbes. However, culturing methodology is still golden standard,
285 as people would ask, if anyone could provide direct evidence for this? Rare study could,
286 because VBNC is nonculturable, mostly it is hard to recover the strain. Also, one
287 important way to control food safety problem, is by accurate detection. Once bacterial
288 cells form VBNC state, the "golden standard" culturing methodology is incapable of
289 detecting, let alone identifying such bacterial cells. However, when we obtain a spoiled
290 food sample (within expiration date, likely to be caused by microbes), how we can
291 confirm it is caused by bacteria in VBNC state, or which bacterial species actually is
292 responsible for this spoilage? Suppose within this spoiled food samples, there are
293 different species, including strain A (both in culturable and VBNC), strain B (both in
294 culturable and VBNC), strain C (only in VBNC), etc. A few methodologies to be used
295 are discussed as follows: 1. If we subject the sample to culturing on an agar plate, partial
296 cells of A and B will be found. However, it is possible that strain C is the responsible
297 spoilage bacteria instead of A and B. 2. If we apply PMA (or other fluorescent dye) with
298 nucleic acid amplification detection, we are able to tell the existence of viable cells, and
299 for the species to be detected, it depends on the primers pairs we use. For example, if

we use PMA-PCR on *femA* gene, positive result could tell there is viable *S. aureus* cells (Jiang et al., 2021). Same for *rfbE* gene on *E. coli* and *invA* gene on *Salmonella* (Zhou et al., 2020; Ou et al., 2021). Another way is, we can use *16S rRNA* gene, however, this could only tell there is viable bacterial cells inside the food samples, but which species still remain unknown. 3. If we use Live/Dead BacLight bacterial viability kit with flow cytometry or microscopy, it only also tells there is viable cells inside, but which species remains unclear.

In this study, we had obtained a spoiled beer samples within expiration date. At the very first, we had no idea what species, how many species and in what state of the microbes. Our approach is, firstly, we used the spoiled food sample for DNA isolation and perform genomic sequencing by Illumina/PacBio. This is the only way to confirm how many and what species are within the food sample. In the example above, via genomic sequencing, we can determine there is strain A or/and B or/and C within. An alternative is *16r RNA* sequencing, however, this can only work on bacteria than fungi, mostly can only identify to the genus level and may raise significant inaccuracy issue. In our study, we had performed genomic sequencing on the beer samples by Illumina. According to the sequencing results, only one distinctive genome as *P. damnosus* was found, which ruled out the possibility of existence of other strains. However, at this point, we were still unable to determine what state the detected microbes are in and the characteristics of the microbes (for example, are they really spoilage bacteria, are they capable of entering VBNC state and cause further food spoilage). Secondly, we had

subjected the spoiled beer sample to culturing on MRS and LB agar plates, followed by CFU counting and routine bacterial identification including morphology observation, biochemistry confirmation and PCR. Thirdly, we had dyed the viable cells using Live/Dead BacLight bacterial viability kit and perform viable cell counting on flow cytometry. Via the difference between viable cells and culturable cells, we further confirmed the approximate number of VBNC cells in the spoiled beer sample. In the example above, combining these 2 methods, it is able to determine the state of existent microbes (like strain A and/or B and/or C). Thirdly, since we had recovered one *P. damnosus* strain from MRS plate, we further subjected this isolate for genomic sequencing, to confirm the recovered strain on the plate and the one in beer sample are identical. Further investigation on this strain was performed. In addition, we had performed VBNC induction to mimic the environments of the beer and the results showed this strain is capable of entering into the VBNC state under beer subculturing and low temperature storage. At last, we had performed spoilage experiment to show this strain is capable of producing different substances to spoil beer sample.

5. Conclusion

In the current study, we used a novel procedure to confirm a beer spoilage case caused by a *Pediococcus damnosus* strain in the VBNC state. A beer spoilage case was first determined to be caused by the co-existence of culturable and VBNC state *P. damnosus* strain BM-PD14610. In addition, VBNC cells were obtained in both

simulation conditions, including beer low temperature storage and beer subculturing. Furthermore, MRS agar supplemented with catalase was found to resuscitate VBNC cells, and normal, VBNC and resuscitated cells showed similar level of beer spoilage capability. This study, and the procedure we had used, will aid in further VBNC study so more evidence on the food safety problem caused by VBNC microbes will be shown. I believe this will further unveil how much of a role VBNC cells are playing in food spoilage and poisoning, as this has posed the critical issue for culturing method thus influences its accuracy.

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Conflict of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

References

Asakura, H., Makino, S., Takagi, T., Kuri, A., Kurazono, T., Watarai, M., & Shirahata, T. (2002). Passage in mice causes a change in the ability of *Salmonella enterica* serovar Oranienburg to survive NaCl osmotic stress: resuscitation from the viable but non-culturable state. *FEMS Microbiology Letters*, 212, 87-93. https://doi.org/10.1016/s0378-1097(02)00711-5.

Behr, J., & Vogel, R.F. (2010). Mechanisms of hop inhibition include the transmembrane redox reaction. *Applied and Environmental Microbiology*, 76, 142-149. https://doi.org/10.1128/AEM.01693-09.

Behr, J., Gessler, A.J., Schmid, J., Zehe, A., & Vogel R.F. (2016). The identification of novel diagnostic marker genes for the detection of beer spoiling *Pediococcus damnosus* strains using the BlAst Diagnostic Gene findEr. *PLoS One*, 11(3), Article e0152747. https://doi.org/10.1371/journal.pone.0152747.

Buddhika, U.V.A. & Seneviratne, G., (2019). Media manipulation with in vitro developed biofilms to cultivate yet-unculturable bacteria from soil. *International Journal of Science Research and Reviews*, 8(4), 377-384. https://doi.org/10.37794/IJSRR.2019.8428.

Cao, X., Zhao, L.C., Zhang, J.F., Chen, X., Shi, L., Fang, X., Xie, H., Chang, Y.L., & Wang, L. (2019). Detection of viable but nonculturable *Vibrio parahaemolyticus* in

shrimp samples using improved real-time PCR and real-time LAMP methods. *Food Control*, 103, 145-152. https://doi.org/10.1016/j.foodcont.2019.04.003.

Chen, H., Zhao, Y.Y., Shu, M., Zhang, T.T., Bi, Y., Gao, Y.Y., & Wu, G.P. (2018). Detection and evaluation of viable but non-culturable *Escherichia coli* O157:H7 induced by low temperature with a BCAC-EMA-Rti-LAMP assay in chicken without enrichment. *Food Analytical Methods*, 12, 458-468. https://doi.org/10.1007/s12161-018-1377-9.

Colwell, R.R., Brayton, P., Herrington, D., Tall, B., Huq, A., & Levine, M.M. (1996). Viable but non-culturable *Vibrio cholerae* O1 revert to a cultivable state in the human intestine. *World Journal Microbiology & Biotechnology*, 12, 28-31. https://doi.org/10.1007/BF00327795.

Deng, Y., Liu, J.Y., Li, L., Fang, H.J., Tu, J.X., Li, B., Liu, J., Li, H.P., & Xu, Z.B. (2015). Reduction and restoration of culturability of beer-stressed and low-temperature-stressed *Lactobacillus acetotolerans* strain 2011-8. *International Journal of Food Microbiology*, 206, 96-101. https://doi.org/10.1016/j.ijfoodmicro.2015.04.046.

Jallouli, W., Zouari, N., & Jaoua, S. (2010). Involvement of oxidative stress and growth at high cell density in the viable but nonculturable state of *Photorhabdus temperata* ssp. *temperata* strain K122. *Process Biochemistry*, 45, 706-713. https://doi.org/10.1016/j.procbio.2010.01.007.

Jiang, H., Wang, K., Yan, M.X., Ye, Q., Lin, X.J., Chen, L., Ye, Y.R., Zhang, L., Liu, JY., & Huang, T.Y. (2021). Pathogenic and virulence factor detection on viable but non-culturable methicillin-resistant *Staphylococcus aureus*. *Frontiers in Microbiology*, 12, Article 630053. https://doi.org/10.3389/fmicb.2021.630053.

Kajala, I., Bergsveinson, J., Friesen, V., Redekop, A., Juvonen, R., Storgards, E., & Ziola, B. (2018). *Lactobacillus backii* and *Pediococcus damnosus* isolated from 170-year-old beer recovered from a shipwreck lack the metabolic activities required to grow in modern lager beer. *FEMS Microbiology Ecology*, 94(1), Article fix152. https://doi.org/10.1093/femsec/fix152.

Kong, H.G., Bae, J.Y., Lee, H.J., Joo, H.J., Jung, E.J., Chung, E., & Lee, S.W. (2014). Induction of the viable but nonculturable state of *Ralstonia solanacearum* by low temperature in the soil microcosm and its resuscitation by catalase. *PloS One*, 9(10), Article e109792. https://doi.org/10.1371/journal.pone.0109792.

Li, Y., Huang, T.Y., Mao, Y.Z., Chen, Y.N., Shi, F., Peng, R.X., Chen, J.X., Bai, C.Y., Chen, L., Wang, K., & Liu, J.Y. (2020). Effect of environmental conditions on the formation of the viable but nonculturable state of *Pediococcus acidilactici* BM-PA17927 and its control and detection in food system. *Frontiers in Microbiology*, 11, Article 586777. https://doi.org/10.3389/fmicb.2020.586777.

Liu, J., Deng, Y., Li, L., Li, B., Li, Y.Y., Zhou, S.S., Shirtliff, M.E., Xu, Z.B., & Peters, B.M. (2018a). Discovery and control of culturable and viable but non-culturable cells of a distinctive *Lactobacillus harbinensis* strain from spoiled beer. *Scientific*

Reports, 8, Article 11446. https://doi.org/10.1038/s41598-018-28949-y.

Liu, J., Deng, Y., Soteyome, T., Li, Y.Y., Su, J.Y., Li, L., Li, B., Shirtliff, M.E., Xu, Z.B. & Peters, B.M. (2018b). Induction and recovery of the viable but nonculturable state of hop-resistance *Lactobacillus brevis*. *Frontiers in Microbiology*, 9, Article 2076. https://doi.org/10.3389/fmicb.2018.02076.

Liu, J., Zhou, R., Li, L., Peters, B.M., Li, B., Lin, C.W., Chuang, T.L., Chen, D.Q., Zhao, X.H., Xiong, Z.Y., Xu, Z.B., & Shirtliff, M.E. (2017a). Viable but non-culturable state and toxin gene expression of enterohemorrhagic *Escherichia coli* O157 under cryopreservation. *Research in Microbiology*, 168, 188-193. https://doi.org/10.1016/j.resmic.2016.11.002.

Liu, J., Li, L., Li, B., Peters, B.M., Deng, Y., Xu, Z.B., & Shirtliff, M.E. (2017b). First study on the formation and resuscitation of viable but nonculturable state and beer spoilage capability of *Lactobacillus lindneri*. *Microbial Pathogenesis*, 107, 219-224. https://doi.org/10.1016/j.micpath.2017.03.043.

Liu, J., Li, L., Peters, B.M., Li, B., Chen, L.Q., Deng, Y., Xu, Z.B., & Shirtliff, M.E. (2017c). The viable but nonculturable state induction and genomic analyses of *Lactobacillus casei* BM-LC14617, a beer-spoilage bacterium. *MicrobiologyOpen*, 6(5), Article e506. https://doi.org/10.1002/mbo3.506.

Liu, J., Li, L., Li, B., Peters, B.M., Deng, Y., Xu, Z.B., & Shirtliff, M.E. (2017d). Study on spoilage capability and VBNC state formation and recovery of *Lactobacillus*

plantarum. *Microbial Pathogenesis*. 110, 257-261. https://doi.org/10.1016/j.micpath.2017.06.044.

Makino, S.I., Kii, T., Asakura, H., Shirahata, T., Ikeda, T., Takeshi, K., & Itoh, K. (2000). Does enterohemorrhagic *Escherichia coli* O157:H7 enter the viable but nonculturable state in salted salmon roe? *Applied and Environment Microbiology*, 66, 5536-5539. https://doi.org/10.1128/AEM.66.12.5536-5539.2000.

Mougin, J., Copin, S., Bojolly, D., Raguenet, V., Robert-Pillot, A., Quilici, M.L., Midelet-Bourdin, G., Grard, T., & Bonnin-Jusserand, M. (2019). Adhesion to stainless steel surfaces and detection of viable but non cultivable cells of *Vibrio parahaemolyticus* and *Vibrio cholerae* isolated from shrimps in seafood processing environments: Stayin' alive? *Food Control*, 102, 122-130. https://doi.org/10.1016/j.foodcont.2019.03.024.

Nicolò, M.S., Gioffre, A., Carnazza, S., Platania, G., Di Silvestro, I., Guglielmino, SPP. (2011). Viable but nonculturable state of foodborne pathogens in grapefruit juice: a study of laboratory. *Foodborne Pathogens and Disease*, 8(1), 11-17. https://doi.org/10.1089/fpd.2009.0491.

Oliver, J.D. (2010). Recent findings on the viable but nonculturable state in pathogenic bacteria. *FEMS Microbiology Reviews*, 34(4), 415-425. https://doi.org/10.1111/j.1574-6976.2009.00200.x.

Ou, A., Wang, K., Ye, Y.R., Chen, L., Gong, X.J., Qian, L., & Liu, J.Y. (2021). Direct

463 detection of viable but non-culturable (VBNC) Salmonella in real food system by
464 a rapid and accurate PMA-CPA technique. *Frontiers in Microbiology*, 12, Article
465 634555. https://doi.org/10.3389/fmicb.2021.634555.

466 Sachidanandham R., & Gin, Y.H. (2009). A dormancy state in nonspore-forming
467 bacteria. *Applied Microbiology and Biotechnology*, 81(5), 927-941.
468 https://doi.org/10.1007/s00253-008-1712-y.

469 Xu, Z., Luo, Y.T., Mao, Y.Z., Peng, R.X., Chen, J.X., Soteyome, T., Bai, C.Y., Chen,
470 L., Liang, Y., & Su, J.Y. (2020). Spoilage lactic acid bacteria in the brewing industry.
471 *Journal of Microbiology and Biotechnology*, 30(7), 955-961.
472 https://doi.org/10.4014/jmb.1908.08069.

473 Yamamoto, H. (2000). Viable but nonculturable state as a general phenomenon of non-
474 sporeforming bacteria, and its modeling. *Journal of Infection and Chemotherapy*,
475 6(2), 112-114. https://doi.org/10.1007/PL00012149.

476 Zerbino, D.R., & Birney, E. (2008). Velvet: algorithms for de novo short read assembly
477 using de Bruijn graphs. *Genome Research*, 18(5), 821-829.
478 https://doi.org/10.1101/gr.074492.107.

479 Zhou, W.Q., Wang, K., Hong, W., Bai, C.Y., Chen, L., Fu, X., Huang, T.Y., & Liu,
480 J.Y. (2020). Development and application of a simple “easy to operate” propidium
481 monoazide-crossing priming amplification on detection of viable and viable but
482 non-culturable cells of O157 *Escherichia coli*. *Frontiers in Microbiology*, 11,

483 Article 569105. https://doi.org/10.3389/fmicb.2020.569105.

Table 1. Organic acids and diacetyl concentrations in *P. damnosus* spoiled beer.

State	Diacetyl (mg/L)	Lactic acid (mg/L)	Acetic acid (mg/L)
Negative control	0.03±0.00 [a]	63.1±13.4 [a]	117.6±28.8 [a]
VBNC	0.07±0.01 [b]	150.0±25.2 [b]	139.3±24.9 [b]
Resuscitated	0.14±0.02 [c]	209.1±48.5 [c]	159.4±39.0 [c]
Logarithmic growing	0.16±0.02 [c]	213.2±33.8 [c]	179.9 ±28.7 [c]

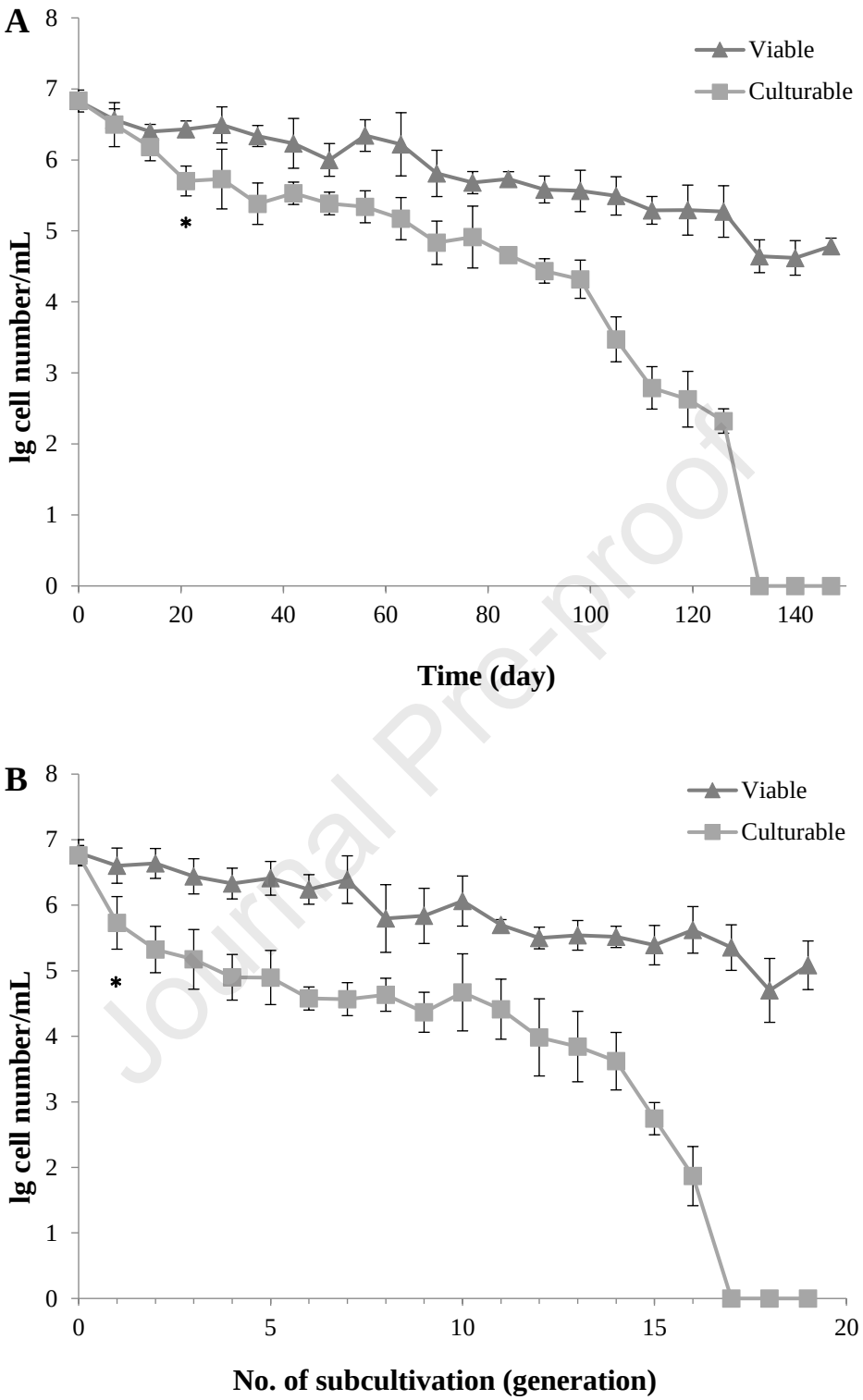

Fig. 1. Viable (▲) and culturable (■) cell numbers during VBNC inducing process in low temperature storage (A) and beer subculturing (B) systems. "*" indicates p value <0.05.

Highlights

- This is the first report on beer spoilage caused by VBNC state *P. damnosus*.
- VBNC state was induced by beer subculturing and low temperature.
- MRS agar supplemented with catalase was found to induce the resuscitation of VBNC cells.
- Normal, VBNC and resuscitated cells showed similar level of beer spoilage capability.

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: