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Characterisitcs of *Saccharomyces boulardii* for reducing ammonia emission from livestock manure

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Abstract

Ammonia from livestock manure acts as a precursor to produce particulate matter (PM) by reacting with atmospheric chemical components volatilized from various sources. Ammonia itself acts as a toxic substance to human health, and thus has direct or indirect adverse effects on human health. This study aimed to verify the effectiveness and mechanism of action of *Saccharomyces boulardii* (SB) in reducing the ammonia emission from livestock manure. The specific ability of SB was confirmed through comparative verification with *S. cerevisiae* (SC) belonging to the same genus. SB and SC could use 50% of ammonia–nitrogen as inorganic nitrogen source in minimal medium. In the control group, the pH level of manure was significantly increased compared to the pH level at 0 h, and the DNA concentration of *Proteus mirabilis*, which increase the manure pH through ammonia production, was found to increase by 2.7-fold. Significant decrease in pH and proliferation of *P. mirabilis* was found in SB group compared to control ($p < 0.05$). The SB group also reduced the amount of ammonia emitted from manure by 25% for 35 days. These results suggested that SB contributed to reducing ammonia emission from manure by reducing pH and inhibiting HAB as well as removing ammonia–nitrogen. Accordingly, SB as a microbiological agent is expected to contribute not only to reduce ammonia emission but also to improve manure quality as a fertilizer.

Keywords: Ammonia emission, Fine dust, Livestock manure, Yeast

Introduction

Ammonia, which acts as a precursor of fine dust, is exhausted in gaseous form and reacts with NO_x and SO_x in the atmosphere to produce ammonium nitrate and ammonium sulfate, respectively, which are particulate fine dusts [1]. The fine dust which is produced by the reaction of ammonia has a longer residence time in the atmosphere than other air pollutants, and thus there is a risk of spreading further causing more serious health problem [1]. Ammonia itself enters the human body and directly causes various diseases through toxic effects. According to the most recent statistical reports from the Ministry of Environment of European Union and South Korea, majority of the ammonia emission is from agricultural sector (> 80%). Of the 80%, livestock manure occupies the largest proportion [2, 3]. Livestock manure is characterized by large quantities of ammonia production and gas emissions through the action of microorganisms having urease and deaminase activities [4–7]. In addition, ammonia reacts with water to increase pH levels by dissociation of hydroxide ions, thereby increasing gaseous ammonia conversion and emission [8, 9].

Research on the management of ammonia gas emission has been conducted worldwide in order to reduce fine dust [10]. Reducing methods, such as acidification, adsorption, and addition of biological materials, as well as physical methods of collecting ammonia gas in livestock facilities, have been described [11, 12]. Among these, various studies employing microorganisms have

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been conducted because the reduction technology using microbes is economical and shows a lower incidence of secondary pollution problems than physical or chemical methods which can cause the secondary pollution problem such as soil acidification and eutrophication. However, most studies have focused on ammonia or liquid manure to reduce odors and harmful gases from the perspective of workers, and there have been insufficient studies concerning atmospheric ammonia emissions and solid manure.

Yeasts of the genus *Saccharomyces* are known to synthesize amino acids using organic and inorganic nitrogen sources [13–15]. Among them, *S. boulardii* (SB) is used as a biological therapeutic agent and a feed additive to increase livestock productivity [16–18]; additionally, it is a probiotic yeast that produces organic acids. Moreover, it has higher stability under acidic and high-temperature conditions compared to *S. cerevisiae* (SC) [19–21]. Furthermore, it has been reported that metabolites of SB inhibit the growth of intestinal pathogenic microorganisms such as *Clostridium difficile* and *Salmonella*, while increasing the growth of beneficial bacteria such as lactic acid bacteria [16, 17, 22]. Given these capacities, SB may have potential efficacy in the inhibition of harmful ammonia-producing bacteria and pathogens. This study intended to confirm the unprecedented application of a biological agent to reduce ammonia emissions by treating livestock manure with SB.

Materials and methods

Manure sample and microorganisms

Manure was collected from a livestock barn in Anseong, Korea. Samples were prepared by mixing 50% swine manure, 10% cattle manure, 10% poultry manure, and 30% sawdust and then used for analysis. All experiments were performed with SB (CNCM I-1079) obtained from LALLEMAND Inc. (Montreal, Canada). SC (KCTC 7107) was purchased from KCTC (Korean Collection for Type Cultures, Daejeon, Korea) and used for comparative analysis with SB belonging to the same genus. *Proteus mirabilis* (KCTC 2510), purchased from KCTC, was used for genomic quantification of ammonia-producing bacteria in manure.

Microbial growth conditions

Yeasts, at an inoculum size of 2% (v/v), were inoculated in yeast extract peptone dextrose (YPD, Difco™, Detroit, MI, USA) medium containing 0.1% (v/v) penicillin–streptomycin solution (Hyclone™, Logan, Utah, USA). The medium was sterilized for 15 min at 121 °C, and yeasts were cultured in a microbial incubator (JSRI-250 T, Gongju, Korea) at 30 °C. *P. mirabilis* was grown in nutrient broth (NB, Difco™) at 37 °C, in a microbial incubator. All microorganisms were used in the experiments after subculturing three times or more.

Analysis of rate of ammonia removal by yeasts in minimal medium

To verify the ammonia removal activity of yeasts in the minimal medium, 2% (w/v) glucose (Oriental Chemical Industries Co., Ltd., Seoul, Korea) was used as the carbon source, and 1% (w/v) ammonium chloride (Fisher Scientific International, Inc., Pittsburgh, Pennsylvania, USA) was used as the nitrogen source. After dissolving glucose and ammonium chloride in yeast nitrogen base (YNB) without amino acids and ammonium sulfate (Difco™), yeasts (SB and SC) were inoculated at an inoculum size of 2% (v/v) and incubated with shaking at 30 °C and 130 rpm for 24 h. The yeast cultures were centrifuged at 4 °C and 10,000 × *g*, and the supernatant was used for the determination of ammonium nitrogen concentration via the indophenol method [23].

DNA extraction and qPCR

DNA extraction was performed using a Fast DNA spin kit for soil (MP Biomedicals, Santa Ana, California, USA), and DNA concentrations were measured using a NanoDrop ND-1000 (Thermo Fisher Scientific, Waltham, Massachusetts, USA). The primers used in this study are listed in Table 1 [24–27]. qPCR analysis was modified by referring to the research of Bokulich et al. [28]. In particular, qPCR amplification was performed in 20 µL final volumes containing 8 µL of DNA (2.5 ng/µL), 1 µL each of forward and reverse primers (10 pmol), and 10 µL of 2 × GoTaq® qPCR Master mix (Promega, Wisconsin, USA). Amplification was carried out using a 7500 Real-Time PCR System (Applied Biosystems, Waltham, MA, USA). The reactions were run for 45 cycles: pre-denaturation at 95 °C for 10 min, denaturation at 95 °C for 15 s, and annealing and extension at 60 °C for 1 min. For melt-curve analysis, the process was performed at 95 °C for 15 s, 60 °C for 1 min, and 95 °C for 30 s. Standard curves for quantifying the amount of microbial DNA were created by plotting the cycle threshold (Ct) values of the qPCR performed against a dilution series of the extracted DNA of the microbial pure cultures (Fig. 1).

Analysis of yeast growth characteristics

For the analysis of growth of SB and SC in livestock manure, 1 g of livestock manure and 5 mL of distilled water were mixed; 160 µL of the pure culture solution was inoculated into this mixture to confirm the change in DNA proportion according to the incubation for 24 h. Aerobic culture conditions were maintained by shaking the culture at 30 °C and 130 rpm, and MGC AnaeroPack

Table 1 List of PCR primer pairs used for qPCR

Target strain	Primer name	Sequence(5′–3′)	Product size (bp)	References
Universal yeast	YEASTF	GAGTCGAGTTGTTTGGGAATGC	124	[24]
	YEASTR	TCTCTTTCCAAAGTTCTTTTCATCTTT		
Saccharomyces cerevisiae	SCDF	AGGAGTGCGGTTCTTTG	310	[25]
	SCDR	TACTTACCGAGGCAAGCTACA		
Total bacteria	TBF	CGGTGAATACGTTCCCGG	142	[26]
	TBR	TACGGCTACCTTGTTACGACTT		
Proteus mirabilis	ureRF	GGTGAGATTTGTATTAATGG	225	[27]
	ureRR	ATAATCTGGAAGATGACGAG		

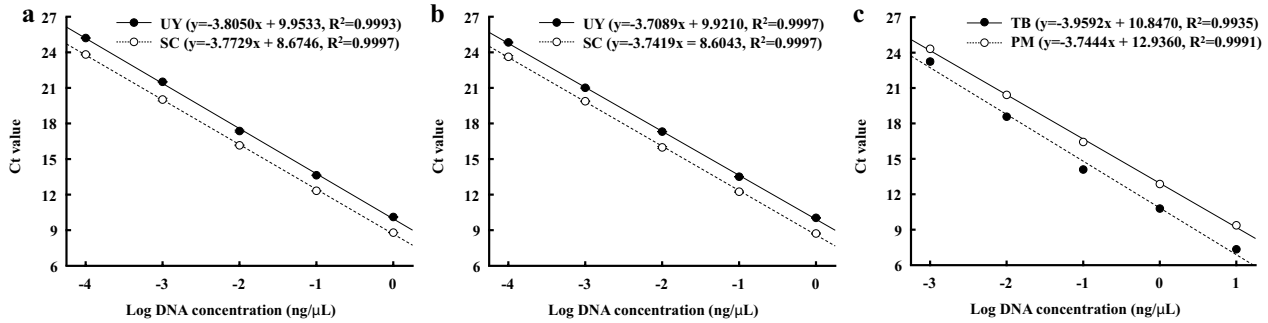

Fig. 1 Linear correlation between the log of genomic DNA concentration and Ct values from qPCR. Each reaction was performed in triplicate. UY, universal yeast; SC, *Saccharomyces cerevisiae*; PM, *Proteus mirabilis*. **a** DNA of *Saccharomyces boulardii* CNCM I-1079 pure culture, **b** DNA of *Saccharomyces cerevisiae* KCTC 7107 pure culture, **c** DNA of *Proteus mirabilis* KCTC 2510 pure culture

(Mitsubishi Gas Chemical Co., Tokyo, Japan) was used at 30 °C under anaerobic condition.

Analysis of changes in pH and growth of hyper ammonia-producing bacteria (HAB) in livestock manure

For analysis of pH change and HAB growth after treatment with SB and SC, 100 µL (6.80 log CFU) of SB and SC pure culture was inoculated into a solution containing 50 mL of distilled water and 10 g of livestock manure. After incubation at 30 °C for 24 h under aerobic and anaerobic conditions, the pH of manure was measured, and the growth of *P. mirabilis* were confirmed through qPCR analysis.

Analysis of ammonia gas emission from livestock manure

Ammonia emissions were analyzed using the air trapping system of Park et al. [29]. For analysis of ammonia gas emissions from livestock manure by treatment of yeast, 100 g of livestock manure sample was inoculated with 1 mL (7.80 log CFU) of yeast (SB and SC) to analyze changes in ammonia emissions resulting from microbial treatment. Ammonia gas emitted in the chamber was transported to a gas bubble-collecting flask containing 50 mL of 0.05 N H_2SO_4 (95%, Dae Jung Chemicals, Gyeonggi-Do, Korea), through air inflow using an air pump and a gas flow meter, and trapped at the same time. Air inflow and outflow rates were maintained at 1 L/min to analyze daily ammonia emissions.

Statistical analysis

The results of each experiment were expressed as the mean ± SEM of triplicate experiments, and statistical analysis was performed using the SAS v.9.4 program (SAS Institute Inc., Cary, North Carolina, USA). Differences between groups were confirmed Student's *t*-test and one-way ANOVA, followed by Tukey's post hoc test with a significance level at $p < 0.05$.

Results and discussion

Ammonia removal abilities of yeasts in minimal medium

Reduction of ammonia gas volatilization and ammonia nitrogen concentrations using microorganisms proceeds via various mechanisms such as nitrification, denitrification, and nitrogen fixation. In addition, the metabolic ability of ammonia nitrogen, which is a source of ammonia gas, is a crucial indicator for suppressing ammonia gas emission [30–32]. SB and SC significantly reduced ammonia nitrogen compared to 0 h

($p < 0.05$) by approximately 50% in YNB medium, which is used as a minimal medium (Fig. 2), and ammonia gas volatilization was not observed in the culture medium during the experiment. This result suggested that ammonia nitrogen was metabolized to non-ammonia nitrogen in the medium without emission in gaseous form; this indicates that both the yeasts can use ammonia nitrogen as an inorganic nitrogen source.

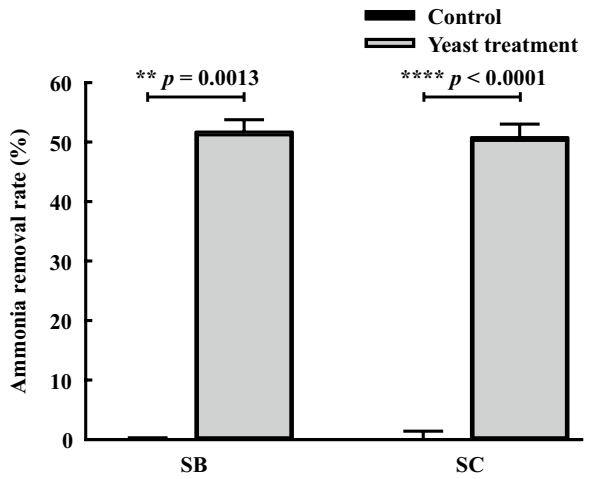

Fig. 2 Ammonia removal rate of SB and SC in YNB medium with NH_4Cl as a nitrogen source in shaking culture (130 rpm). SB, Saccharomyces boulardii CNCM I-1079; SC, Saccharomyces cerevisiae KCTC 7107. Error bars represent the standard error (n = 3). **p < 0.01, ****p < 0.0001, compared between control and yeast treatment groups, indicates statistical significance (SB treatment vs. control: f-value = 52.41, degree of freedom (DF) = 2.0763, t-value = − 24.95, p = 0.0013; SC treatment vs. control: f-value = 2.50, DF = 4, t-value = −19.03, p < 0.0001; SB vs SC treatments: f-value = 1.21, DF = 4, t-value = 0.31, p = 0.7749)

Growth characteristics of yeasts in manure depending on culture conditions

The growth of yeasts depending on the presence of oxygen in livestock manure was confirmed based on the change in the DNA ratio of both SB and SC to total yeasts (Fig. 3). *Saccharomyces* spp. strains are facultative anaerobic microbes capable of proliferating under both aerobic and anaerobic conditions [33, 34], and it was confirmed that SB and SC could grow in livestock manure under all tested conditions. After 24 h incubation, DNA proportion of SB increased from approximately 20–45% under all culture conditions (SB in aerobic condition, $p = 0.0419$; SB in anaerobic condition, $p = 0.0481$). Additionally, the DNA proportion of SC increase from 10 to 47% under aerobic condition and from 17 to 50% under anaerobic condition (SC in aerobic condition, $p = 0.0002$; SC in anaerobic condition, $p = 0.0256$). In the case of both SB and SC, it is expected that their growth in manure environment is possible, considering that the dominance increased 2–4 times compared to 0 h in the manure medium conditions.

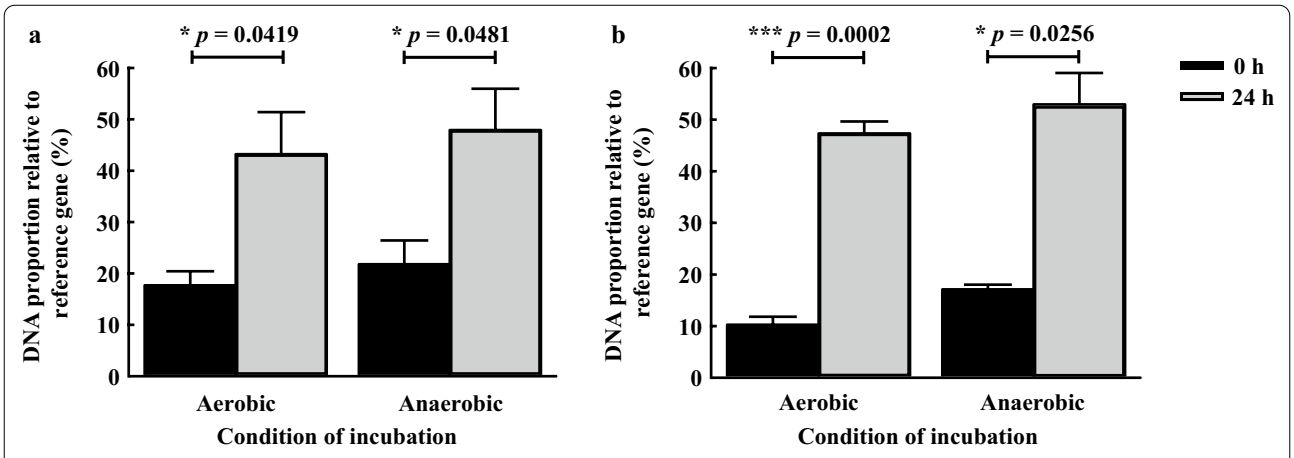

Fig. 3 Quantitative real-time PCR analysis to determine the quantity of yeast genomic DNA content relative to a universal gene after 24 h of incubation for analysis of DNA proportions in manure. The strains were incubated under aerobic and anaerobic conditions at 30 °C. Values are the mean ± SEM. Error bars represent the standard error (n = 3). **a** DNA proportion of *Saccharomyces boulardii* CNCM I-1079 (SB), **b** DNA proportion of *Saccharomyces cerevisiae* KCTC 7107 (SC). *$p < 0.05$, ***$p < 0.001$, compared between 0 and 24 h DNA proportion in the same group and same condition, indicates statistical significance (0 vs. 24 h of SB in the aerobic condition: f-value = 9.00, degree of freedom (DF) = 4, t-value = − 2.95, $p = 0.0419$; 0 vs. 24 h of SB in the anaerobic condition: f-value = 2.98, DF = 4, t-value = − 2.81, $p = 0.0481$; 0 vs. 24 h of SC in the aerobic condition: f-value = 2.22, DF = 4, t-value = − 13.28, $p = 0.0002$; 0 vs. 24 h of SC in the anaerobic condition: f-value = 44.65, DF = 2.0895, t-value = − 5.81, $p = 0.0256$)

Changes in pH and growth of HAB in manure by yeast treatment

Increased ammonia concentrations enhance the pH by the dissociation of hydroxide ions, and the ionic and gaseous forms of ammonia are determined by changes of the pH. For this reason, changes in ammonia concentrations and pH are substantial causes of ammonia gas emissions [8, 9]. It has been reported that ammonia concentrations can be controlled by using oxidizing agents, such as iron chloride, sulfuric acid [35, 36], or various chemical compounds [5] that reduce ammonia emission from livestock manure. Furthermore, the effects of SB and SC in manure were evaluated as they possess activities of pathogen inhibition by antimicrobial peptide production and pH reduction [21, 37]. Indeed, *Saccharomyces* spp. generally produce organic acids during growth [38].

For more accurate observations of the changes in manure due to yeast growth, pH levels were measured after 24 h of incubation with liquid livestock manure under aerobic and anaerobic conditions (Table 2). The control ($p = 0.0255$ compared to pH at 0 h) and SC groups ($p = 0.0340$ compared to pH at 0 h) under aerobic condition showed a significant increase in pH from the initial level of 7.34 to more than 7.40 (pH 7.63 for control and pH 7.43 for SC), and the SB group exhibited a tendency to decrease pH by 7.14 compared to 0 h, showing a significant difference ($p < 0.05$) compared with the control and SC groups. In the case of anaerobic condition, all groups demonstrated decreased pH after 24 h. In particular, the SB group showed a significant decrease ($p < 0.05$) in pH by 6.68 compared with the other groups (pH 6.92 for control and pH 6.83 for SC).

It was deduced that the pH change pattern was reversed in the aerobic and anaerobic control groups because of the influence of microorganisms in livestock manure that increased the pH under aerobic condition. Vince et al. [6] reported that the representative aerobic gram-negative bacteria (*Proteus* spp., *Escherichia coli*, etc.), which produce ammonia in livestock manure, accumulate ammonia in the manure by urea hydrolysis and deamination, and these activities are affected by urea concentration and pH change. According to Vince et al. [6], *P. mirabilis* has been reported to produce more than 50 ppm of ammonia per 100 ppm of urea as a substrate. Likewise, it was found in our study that *P. mirabilis*, one of HAB strains analyzed in this study, could not grow under anaerobic condition but could proliferate under aerobic condition; 2.7-fold increase compared to initial DNA concentration in the control group (Fig. 4). In the SB group, *P. mirabilis* showed 1.2-fold increase in DNA concentration compared to the 0 h, and the increase rate in DNA was significantly reduced compared to the

Table 2 Change of pH in manure by using 2 yeasts in aerobic and anaerobic conditions

Time (h)		0		24	
Condition	Group	Average	SE	Average	SE
Aerobic	Control	7.34	0.01	7.63$^{*\,a}$	0.05
	SB	7.34	0.01	7.14^{b}	0.10
	SC	7.34	0.01	7.46$^{*\,a}$	0.02
Anaerobic	Control	7.34	0.01	6.92$^{**\,a}$	0.02
	SB	7.34	0.01	6.68$^{**\,b}$	0.07
	SC	7.34	0.02	6.83$^{***\,a}$	0.01

SB, *Saccharomyces boulardii* CNCM I-1079; SC, *Saccharomyces cerevisiae* KCTC 7107

Values are the mean ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. 0 h indicates statistical significance (0 vs. 24 h of control in the aerobic condition: degree of freedom (DF) = 2, t-value = − 6.14, $p = 0.0255$; 0 vs. 24 h of SB in the aerobic condition: DF = 2, t-value = 1.98, $p = 0.1864$; 0 vs. 24 h of SC in the aerobic condition: DF = 2, t-value = − 5.29, $p = 0.0340$; 0 vs. 24 h of control in the anaerobic condition: DF = 2, t-value = 25.40, $p = 0.0015$; 0 vs. 24 h of SB in the anaerobic condition: DF = 2, t-value = 10.06, $p = 0.0097$; 0 vs. 24 h of SC in the anaerobic condition: DF = 2, t-value = 51.00, $p = 0.0004$. The different letters (a, b) indicate statistically different groups at the same time and under the same conditions (significance level at $p < 0.05$). There were significant differences in pH between three groups in the aerobic condition [F(2, 6) = 14.50, $p = 0.0050$] and anaerobic condition [F(2, 6) = 9.19, $p = 0.0149$]

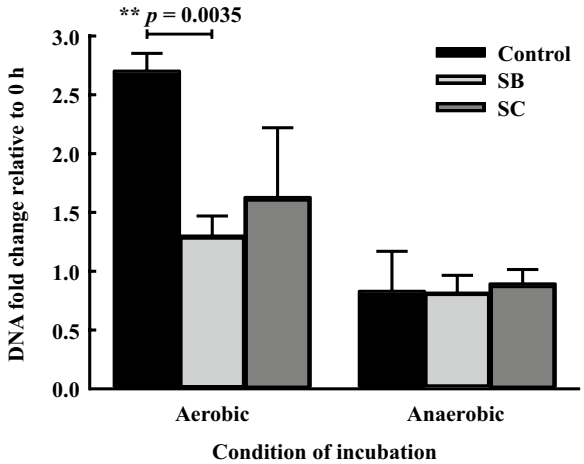

Fig. 4 Real-time PCR analysis to determine the change in genomic DNA concentration of *Proteus mirabilis* (*P. mirabilis*), which is a hyper ammonia-producing bacterium (HAB), in livestock manure by treatment with yeasts. SB, *Saccharomyces boulardii* CNCM I-1079; SC, *Saccharomyces cerevisiae* KCTC 7107. Values on the y-axis represent fold change in DNA levels at 24 h compared to DNA concentration at 0 h. Error bars represent the standard error (n = 3). $^{**}p < 0.01$, compared between control and SB in the aerobic condition, indicates statistical significance (control vs. SB in the aerobic condition: f-value = 1.27, degree of freedom (DF) = 4, t-value = 6.17, $p = 0.0035$; control vs. SC in the aerobic condition: f-value = 15.44, DF = 4, t-value = 1.76, $p = 0.1537$; control vs. SB in the anaerobic condition: f-value = 5.05, DF = 4, t-value = 0.04, $p = 0.9686$; control vs. SC in the anaerobic condition: f-value = 7.74, DF = 4, t-value = − 0.17, $p = 0.8702$)

control group ($p < 0.05$). In the SC group, DNA concentration of *P. mirabilis* showed 1.7-fold increase, showing a lower increase rate than control, but there was no significant difference compared to the control ($p = 0.1537$). In addition, the inhibitory effect of HAB under aerobic condition had the same significance as the pH results (Table 2) under aerobic condition.

It has been reported that *Saccharomyces* spp. strains produce organic acids during their growth [38]. However, as the acid-producing ability of SB was superior to that of SC [21], it seemed that the SB group had significantly decreased the pH of the manure compared to other groups, under all conditions, and was highly effective in the inhibition of *P. mirabilis*.

This suggests that pH changes in livestock manure are not only determined by the chemical actions of the manure constituents, but also by the growth and activities of microorganisms inducing environmental changes.

Changes in ammonia gas emission from manure by yeast treatment

The emission characteristics of ammonia gas in manure by yeast treatment were analyzed by the acid trapping of ammonia among the volatile gases and the indophenol method (Fig. 5). Ammonia emission patterns were similar to those of typical emissions from soil fertilized with urea nitrogen [29, 39]. The cumulative ammonia emissions during the analysis period were 841.43 ± 38.36, 642.99 ± 45.91, and 829.27 ± 56.80 mg/kg in the control, SB, and SC groups, respectively, while the SB group significantly reduced ammonia gas emissions by about 25% compared to the control ($p < 0.05$). Conversely, the treatment of SC compared with SB did not show any reduction effects. It was considered that the pH reduction and growth inhibition of SB against *P. mirabilis*, an aerobic ammonia-producing bacterium, positively affected reductions in ammonia gas emissions.

Ammonia emission is difficult to be directly compared due to various influence by environmental conditions such as manure composition, water content, temperature, and microflora. According to the study of Park et al. [29], however, it was reported that the amount of ammonia produced by urea fertilization in the soil decreased in proportion to the amount of mixed microbial treatment. As such, studies related to ammonia emission reduction by microbial treatment have been recently reported [29, 32], and in this aspect of utility, this study attempted to predict the reduction mechanism by effective single microbial treatment. Furthermore, since SB in this study is *Saccharomyces* spp., which is used as a probiotic for livestock, it may be present in feces through ingestion of animals. Therefore, we suggest that SB could not only increases livestock productivity but also reduces ammonia emissions.

Recently, fine dust has become an increasingly serious problem, and many studies have been reported regarding microorganisms that can effectively reduce ammonia emissions. However, most studies have focused on nitrogen conversion by microbial metabolism [30–32], and there has been insufficient research regarding the characteristics of microbial ammonia gas production. In summary, this study clarified the possibility that SB

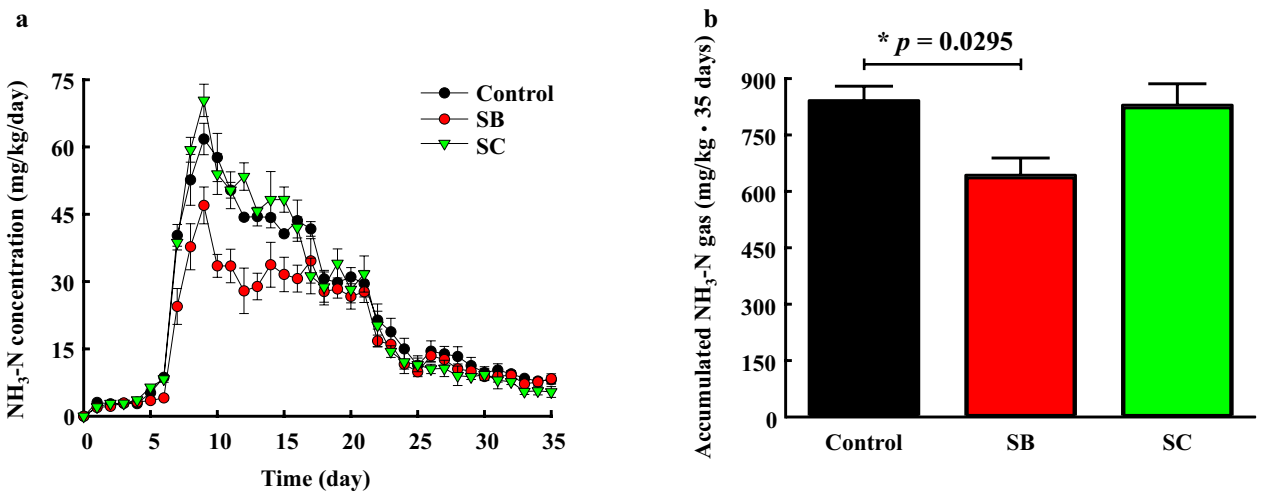

Fig. 5 Changes in NH_3 gas emissions and accumulated ammonia gas in 100 g of livestock manure by treatment with yeasts. SB, *Saccharomyces boulardii* CNCM I-1079; SC, *Saccharomyces cerevisiae* KCTC 7107. Error bars represent the standard error (n = 3). **a** Daily amount of ammonia gas emissions, **b** accumulated amount of ammonia gas over 35 days. *$p < 0.05$, compared between control and SB groups, indicates statistical significance (control vs. SB: f-value = 1.43, degree of freedom (DF) = 4, t-value = 3.32, $p = 0.0295$; control vs. SC: f-value = 2.19, DF = 4, t-value = 0.18, $p = 0.8678$)

can have positive effects on ammonia emission reduction by utilization of ammonia and the repression of environmental factors that affect ammonia emission. Furthermore, if observation on the changing factors is accompanied, it is expected that it can contributed to the research on reducing ammonia emission through microbial inhibitors and broadening the scope of utilization of SB which is already being used for industrial purposes.

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Authors' contributions
S-IK, WH, and Y-JK designed and conducted the experiment as well as wrote the manuscript. S-JL analyzed changes of pH in the manure by microbial treatment. B-KH and H-GL inspired the overall research and revised the final manuscript. All authors read and approved the final manuscript.

Competing interests
The authors declare that they have no competing interests.

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