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Short Communication

Vibrio proteolyticus No. 442, a potential probiotic for tiger puffer, *Takifugu rubripes*

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Abstract: The efficacy of *Vibrio proteolyticus* No. 442 as a probiotic in preventing opportunistic infections caused by Vibrionaceae in tiger puffer, *Takifugu rubripes*, was evaluated. Two strains of *Listonella anguillarum*, IFO 13266^T and Obama 5, were used as target bacteria. The antimicrobial activity of *V. proteolyticus* No. 442 peaks at approximately 25°C and is absent at temperatures at 15°C or above 35°C. This activity is also most pronounced at an oxygen concentration of 21% and is undetectable under anaerobic conditions. Using these characteristics, *V. proteolyticus* No. 442 and *L. anguillarum* IFO 13266 were co-cultured under aerobic and anaerobic conditions. The findings indicated that under aerobic conditions, *L. anguillarum* IFO 13266 was eradicated following a 48-hour incubation at 25°C, while under anaerobic conditions, both species maintained high bacterial densities. Consequently, the oral administration of the diets supplemented with *V. proteolyticus* No. 442 to juvenile tiger puffers for seven days resulted in a decrease in the density of Vibrionaceae (excluding *V. proteolyticus*) by two to three orders of magnitude in the gut, and by one to two orders of magnitude in the rearing water. Future research needs to investigate the long-term efficacy of *V. proteolyticus* as a probiotic, and its pathogenicity, fish growth, administration methods, and storage strategies.

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Introduction

With the rapid increase in population on Earth, there has been a scramble for resources such as food, water, and energy. Fish farming, in particular, is gaining attention as a valuable source of protein. Typically, fish farming involves raising fish at high densities in limited spaces, which often leads to outbreaks of infections from opportunistic bacteria entering through wounds caused by abrasions among the fish. Therefore, representative opportunistic infections, vibriosis, caused by Vibrionaceae are an important risk factor in many fish farming facilities. Once a bacterial infection occurs, it is common to administer antibiotics or other drugs mixed with feeds or through medicated baths. However, there are concerns that the release of these drugs into the surrounding waters of the farms may reduce the purification capacity of the surrounding waters or lead to the emergence of drug-resistant bacteria (Midlen and Redding, 1998; Sugita et al., 2002). As a result, in recent years, probiotics, which are live bacteria that maintain the host's health when administered in appropriate amounts, have gained attention as an alternative to traditional chemotherapy, and many research papers have been published on the subject.

Sugita et al. (2002) isolated bacteria that demonstrated antimicrobial activity against typical fish pathogens, including *Listonella anguillarum* IFO13266, *Vibrio vulnificus* RIMD 2219009, *Photobacterium damselae* subsp. *piscicida* K-III, and *Lactococcus garvieae* ATCC 49156, during the development of Japanese flounder, *Paralichthys olivaceus*. The bacterium with the highest activity was identified as *Vibrio proteolyticus* No. 442. Additionally, this bacterium is characterized by its high chitinase activity (Itoi et al., 2007). In this study, *V. proteolyticus* No. 442 was co-cultured with *L. anguillarum* and then orally administered to

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juvenile tiger puffer fish to evaluate its potential as a probiotic for this fish species.

Materials and Methods

Assay of antibacterial activity: The antimicrobial activity of bacterial cultures was measured using the agar well diffusion method (Balouiri et al., 2016). Two target bacteria of *L. anguillarum,* IFO13266 and Obama 5, were each incubated at 25°C for 24 hours in 1/5 PYBG liquid medium (Sugita et al., 2002). The culture was then diluted to achieve an optical density at 610 nm (OD_{610}) of 0.2, corresponding to approximately 10^8 CFU (colony forming units)/mL, and further diluted 100-fold in 1/5 PYBG soft agar medium (1.0% agar). A volume of 4.5 mL of this diluted culture was layered onto 1/5 PYBG agar plates (1.5% agar). A hole with a diameter of 4 mm was then punched into the agar plate using a sterile cork borer to create the assay plate.

Vibrio proteolyticus No. 442 was pre-incubated at 25°C for 24 hours in PYBG liquid medium (Sugita and Ito, 2006). Ten μL aliquots of the culture were then inoculated into 10 mL of fresh PYBG liquid medium and incubated at 25°C for 48 hours under atmospheric conditions in anaerobic jars with oxygen concentrations ranging from 0-21% (Sugita et al., 1988). Additionally, the culture was incubated at temperatures between 15-40°C for 48 hours under aerobic conditions.

Meanwhile, the culture of *V. proteolyticus* No. 442 was centrifuged twice at 10,000 × g for 10 minutes each at 0°C, and the resulting supernatant was filtered through a 0.2 μm membrane filter (Gelman Science, Ann Arbor, MI). Subsequently, 50 μL of the filtrate was dispensed into holes in assay plates. After incubating at 25°C for 24 hours, the diameter of the clear zone around each hole was measured to determine the antimicrobial activity, expressed in millimeters (mm).

Co-cultivation of *V. proteolyticus* No. 442 and *L. anguillarum* IFO 13266: The conditions of the experiment were determined based on the characteristics of *V. proteolyticus* No. 442, which does not produce antimicrobial agents at both 15°C and above 35°C, as well as under anaerobic conditions. Two sets of bottles were prepared, each containing 100 mL of PYBG liquid medium (100% artificial seawater) within 200 mL medium bottles. The co-cultivation of *V. proteolyticus* No. 442 and *L. anguillarum* IFO 13266 was conducted under aerobic and anaerobic conditions. The strains were pre-cultured twice prior to the experiment, then centrifuged at 10,000×g for 10 minutes at 5°C, and washed twice with fresh PYBG liquid medium. *Vibrio proteolyticus* No. 442 and *L. anguillarum* IFO 13266 were inoculated to achieve densities of 10^3 and 10^4 CFU/mL, respectively. The culture was incubated at 25°C, and aliquots were taken at 0-, 24-, 48-, and 72-hours post-culturing, which were serially diluted and inoculated onto two sets of TCBS agar medium (Eiken Chemical Co., Tokyo). Each inoculated agar plate was further incubated aerobically under two conditions; one at 15°C for 5 days and the other at 35°C for 3 days. The densities of *L. anguillarum* (yellow colonies) and *V. proteolyticus* (green colonies) were determined at each temperature, with the higher density considered the true density. Additionally, the antibacterial activities of the cultures were assessed as described above.

Administration of *V. proteolyticus* No.442 to tiger puffer: After juvenile tiger puffer were raised in an 800 L tank, six tiger puffer each (18.3±4.0 g, body weight) were transferred to five 46 L glass tanks with recirculating water systems at 20±1°C. Artificial seawater (Rei-sea salt G, Iwaki Co., Tokyo) with an average of 31.1±0.7 psu was used as the rearing water for tiger puffer. One week before the start of the experiment, all the water in the tanks was replaced with fresh artificial water. Soft series D pelleted diets (Sakamoto Feeds Co., Chiba) were used to feed at 2% of the body weight to the tiger puffer in each tank**.** After 7 days of feeding the pufferfish with their regular diets, they were fed the same diets but supplemented with *V. proteolyticus* No. 442 (1.0×10^{10} CFU/g) for an additional seven days. Following this, they were returned to their regular diets. The day before and 7th day after administering the probiotic diet, the day after returning to the regular diet, and 2

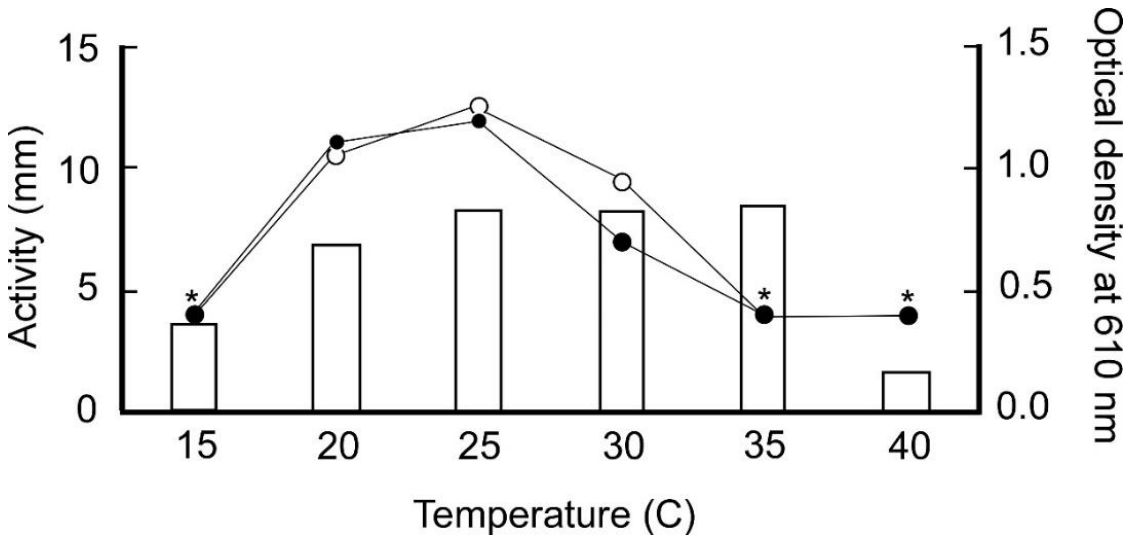

Figure 1. Antibacterial activities against *Listonella anguillarum* IFO 13266 (○) and Obama 5 (●) and optical density at 610 nm (bar) in the culture of *Vibrio proteolyticus* No. 442, incubated at different temperatures ranging from 15 to 40˚C. The asterisk (*) represents "not detected".

days later were designated as day 0, day 7, day 8, and day 10, respectively. All fish specimens were euthanized by immersion in an ice slurry immediately after collection and treated as follows: the gut contents were obtained aseptically by dissection and gentle squeezing. The rearing water was also collected at the same time.

After being diluted serially, the gut contents and rearing water of tiger puffer were inoculated onto TCBS medium and VP8 medium (Muniesa-Pérez et al., 1996), and incubated for three days at 25 and 35°C under aerobic conditions, respectively. The green colonies on the TCBS medium and yellow colonies on the VP8 medium were examined by polymerase chain reaction (PCR) using a *V. proteolyticus*-specific primer, V3VPR (5′-CGC TAA CGT CAA ATA ATG CAT CTA-3′; Muniesa-Pérez et al., 1996), and a universal primer, 20F (5′-AGA GTT TGA TCC TGG CTC AG-3′). The amplification of an approximately 493 bp DNA fragment confirmed it to be *V. proteolyticus*.

Results and Discussions

Effect of temperature and oxygen concentrations on antibacterial activity: Figure 1 shows the antimicrobial activity and OD_{610} of *V. proteolyticus* No. 442 when cultured under aerobic conditions (21% O_2) at 15-40°C for 48 hours. The OD_{610} peaked at 25-35°C, with values at 15 and 40°C reaching 43.5 and 19.8% of the peak value at 35°C, respectively, indicating that the temperature significantly influenced its growth. In addition, the antimicrobial activity was highest at 25°C and undetectable at 15°C and 35-40°C. The activity against *L. anguillarum* IFO 13266 was slightly higher than that against the Obama 5 strain, but the temperature effect was similar for both. These results indicate that the antimicrobial activity of *V. proteolyticus* No. 442 peaks at 25-35°C and is undetected at 15°C and above 35°C.

Figure 2 presents the antimicrobial activity and OD_{610} of *V. proteolyticus* No. 442 cultured at 25°C for 48 hours under varying oxygen concentrations (0-21%). The OD_{610} reached its maximum within the 5-21% O_2 range. Notably, under anaerobic conditions (0% O_2), the OD_{610} was 55.8% of the value at 21% O_2, indicating that oxygen levels significantly influence the growth of this bacterium. The highest antimicrobial activity was observed at 21% O_2, with no activity detected under anaerobic conditions. The antibacterial effect on *L. anguillarum* IFO 13266 was slightly greater than on the Obama 5 strain, but the influence of oxygen concentration was nearly identical for both. These findings demonstrate that *V. proteolyticus* No. 442 requires O_2 for growth and the production of antimicrobial agents.

Co-cultivation of *V. proteolyticus* No. 442 and

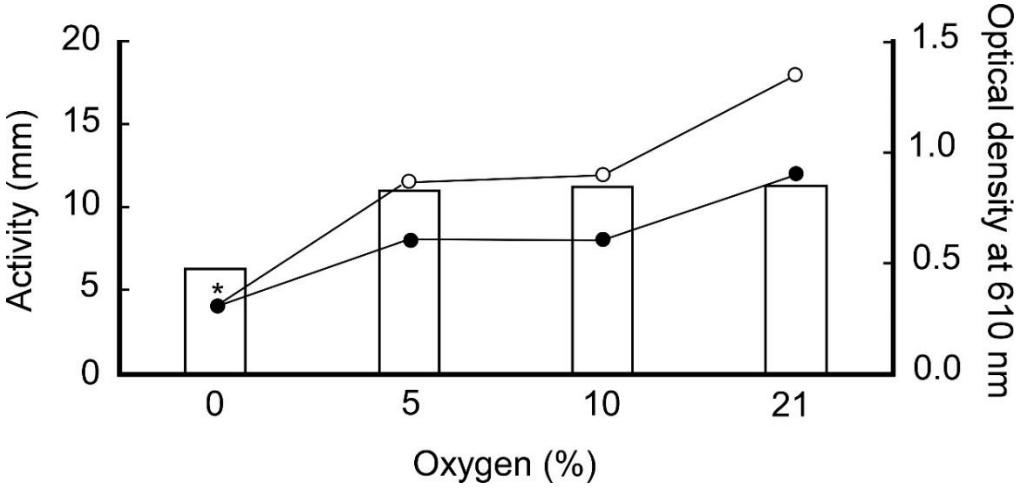

Figure 2. Antibacterial activities against *Listonella anguillarum* IFO 13266 (○) and Obama 5 (●) and optical density at 610 nm (bar) in the culture of *Vibrio proteolyticus* No. 442, incubated at different concentrations of oxygen ranging from 0 to 21%. The asterisk (*) represents "not detected".

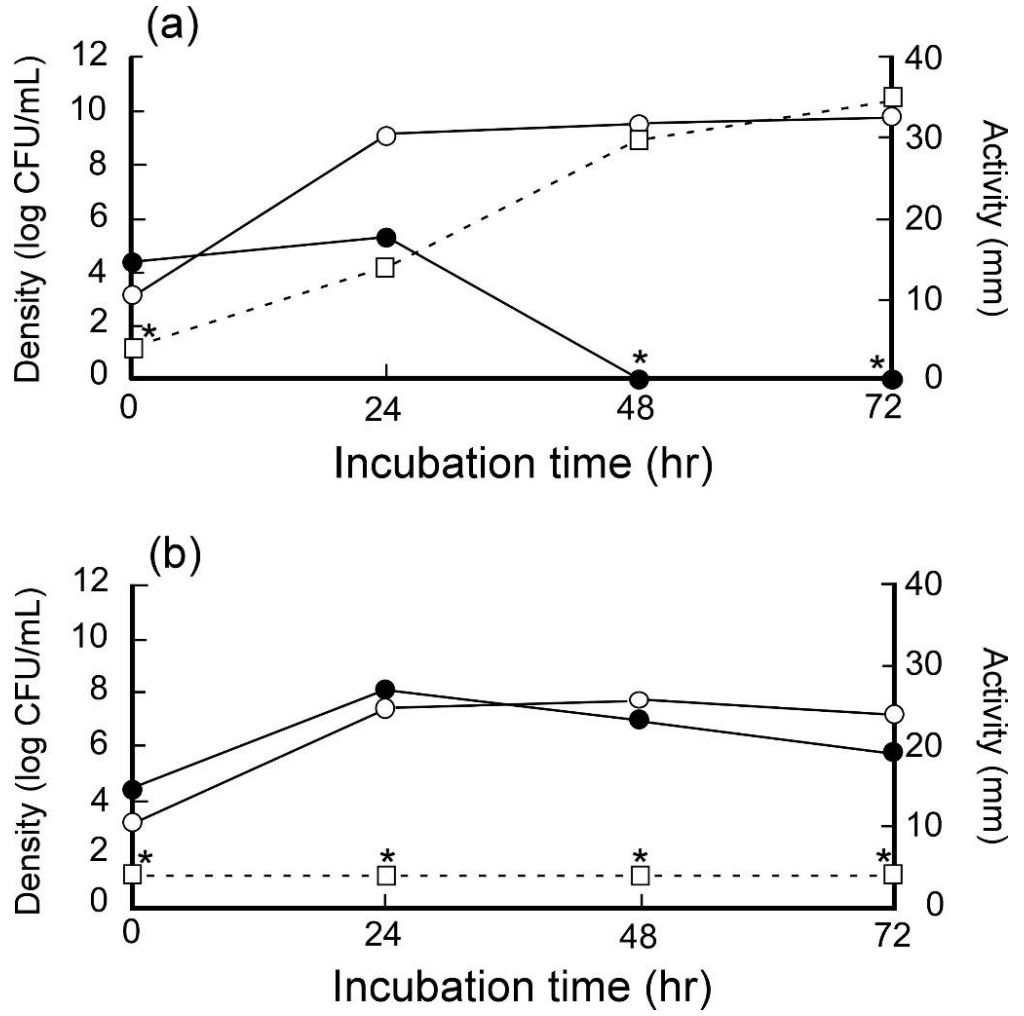

Figure 3. Time-courses of the density of *Vibrio proteolyticus* No. 442 (○), *Listonella anguillarum* IFO13266 (●), and antibacterial activity (□) in their co-culture, incubated under aerobic (a) and anaerobic (b) conditions. The asterisk (*) represents "not detected".

***L. anguillarum* IFO 13266:** In the previous section, we found that *V. proteolyticus* No. 442 does not produce antimicrobial agents under anaerobic conditions. As a result, we decided to conduct co-

cultivation under aerobic and anaerobic conditions to examine the effects of the presence or absence of antimicrobial agents. Figure 3a illustrates changes in the viable count of bacteria and antimicrobial activity in the culture fluid when *V. proteolyticus* No. 442 and *L. anguillarum* IFO 13266 were co-cultured under aerobic conditions. Initially, *V. proteolyticus* No. 442 had a density of 1.4×10^3 CFU/mL, which increased to 1.2×10^9 CFU/mL at 24 hours and further to 5.6×10^9 CFU/mL at 72 hours. Antimicrobial activity was absent at 0 hour, but zones of inhibition measuring 14, 30, and 35 mm at 24, 48, and 72 hours, respectively. Conversely, *L. anguillarum* IFO 13266 started at a higher density of 2.4×10^4 CFU/mL, increased to 2.0×10^5 CFU/mL at 24 hours, and subsequently became undetectable. This disappearance is perhaps attributed to the inhibitory effects of the increasing antimicrobial activity observed after 48 hours.

In contrast, Fig. 3b presents the results of the anaerobic cultivation. *Vibrio proteolyticus* No. 442 started at 1.4×10^3 CFU/mL, with its growth rate diminishing compared to aerobic conditions, peaking at 2.6×10^7 CFU/mL at 24 hours, then slightly declining at 72 hours. No antimicrobial activity was detected from 0 to 72 hours, confirming that *V. proteolyticus* No. 442 does not produce antimicrobial agents under anaerobic conditions. Meanwhile, *L. anguillarum* IFO 13266 began at 2.4×10^4 CFU/mL, surged to 1.8×10^8 CFU/mL at 24 hours, and gradually decreased. Thus, Figures 3 demonstrate that the antibacterial agents produced by *V. proteolyticus* No. 442 effectively inhibit the growth of *L. anguillarum* IFO 13266.

Administration of *V. proteolyticus* to tiger puffer: Tiger puffer was fed a feed containing *V. proteolyticus* No. 442 as a probiotic for seven days. On days 0, 7, 8, and 10, one pufferfish, along with the rearing water, was taken from each tank, and the bacterial density in the gut contents and rearing water was measured. In gut contents, *V. proteolyticus* was not detected on day 0 ($<2\times10^2$ CFU/g), but reached 4.9×10^6 to 7.9×10^8 CFU/g on day 7 (Table 1). The density of Vibrionaceae (excluding *V. proteolyticus*) was 1.7×10^8 to 4.4×10^9 CFU/g on day 0, but decreased to 1.0×10^5 to 6.0×10^7 CFU/g on day 7. Conversely, in the rearing water, *V. proteolyticus* was not detected in all tanks on day 0 ($<2\times10^1$ CFU/mL) but was 1.0×10^5 to 4.8×10^5 CFU/mL on day 7. The density of Vibrionaceae (excluding *V. proteolyticus*) was 4.7×10^5 to 1.4×10^6 CFU/mL on day 0 but decreased to 1.2×10^3 to 1.0×10^5 CFU/mL on day 7. These results indicate that in pufferfish orally administered with *V. proteolyticus* for seven days, the density of Vibrionaeceae (excluding *V. proteolyticus*) decreased by two to three orders of magnitude in the gut and one to two orders of magnitude in the rearing water.

Nogami and Maeda (1992) reported that the occurrence of vibriosis in the rearing of swimming crab, *Portunus trituberculatus*, larvae could be controlled by directly introducing PM-4 bacteria, which have antimicrobial activity against *Vibrio*, into the rearing water. This pioneering study required the daily addition of the PM-4 concentrate to the rearing water of the crab larvae to maintain high densities of inhibitory bacteria, making it difficult to implement in small-scale aquaculture facilities. Therefore, we proposed that a strategy of using the gut of marine organisms as a fermentation tank to supply probiotics, which then proliferate in the rearing water after being excreted with feces, would reduce the density of *Vibrio* in the rearing water and lower the risk of opportunistic infections (Sugita, 2022). Sugita et al. (2009) previously reported that orally administering *Lactococcus lactis* as a probiotic to goldfish *Carassius auratus* reduced the density of *Aeromonas* in the rearing water.

In this study, we experimentally demonstrated that administering *V. proteolyticus* No. 442 to pufferfish reduced the density of Vibrionaceae (excluding *V. proteolyticus*) in the gut contents and rearing water by two to three and one to two orders of magnitude, respectively (Table 1). Although *V. proteolyticus* No. 442 effectively inhibited the growth of *L. anguillarum* IFO 13266 *in vitro*, it could not completely suppress Vibrionaceae in both the gut contents and the rearing water of tiger puffer. This suggests that probiotics alone are ineffective and may be more effective when combined with other husbandry techniques, such as

Table 1. Densities of *Vibrio proteolyticus* No. 442 and Vibrionaceae (excluding *V. proteolyticus*) in the guts and rearing water of tiger puffer administered with *V. ptoteolyticus* No. 442 as probiotic.

No. tank	Day	Guts (log CFU/g)		Rearing water (log CFU/mL)	
		No. 442	Vibrionaceae	No. 442	Vibrionaceae
1	0	nd*	8.52	nd	5.67
	7	6.69	5.00	5.00	3.08
	8	7.00	5.30	4.30	4.70
	10	6.40	8.31	1.70	4.35
2	0	nd	8.81	nd	6.15
	7	7.00	6.54	5.30	3.65
	8	6.41	6.46	5.00	5.00
	10	6.89	9.42	3.85	5.01
3	0	nd	9.65	nd	5.99
	7	7.66	6.00	5.30	5.00
	8	8.22	8.71	4.20	4.35
	10	6.66	9.15	3.40	4.83
4	0	nd	9.57	nd	5.72
	7	8.90	7.78	5.68	4.00
	8	6.53	6.24	5.30	5.00
	10	5.91	6.85	3.33	3.64
5	0	nd	8.23	nd	5.67
	7	7.76	6.48	5.00	4.48
	8	6.81	7.38	5.48	4.00
	10	3.88	7.41	2.30	5.30

*nd, not detected.

hygiene management. Since the administration period was seven days in the current study, further experiments will be necessary to determine the probiotic effects on pufferfish. Recently, Medina et al. (2023) reported that Senegalese sole, *Solea senegalensis* can withstand challenge tests using *Photobacterium damselae* subsp. *piscicida* and *V. harveyi* when *V. proteolyticus* DCF12.2 was administered as a probiotic. According to Buller (2014), *V. proteolyticus* is listed as a pathogen of crustaceans. Our study (Itoi et al., 2007) also suggests that *V. proteolyticus* No. 442, which produces very potent chitinase, could be effective in aiding the digestion of crustacean shells when used for fish but may not be suitable as probiotics for crustaceans. Future research should investigate the long-term efficacy of *V. proteolyticus* as a probiotic, alongside its pathogenicity, fish growth, administration methods, and storage strategies.

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