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IN VITRO ANTAGONISTIC EFFECT OF GRAM-NEGATIVE PROBIOTIC BACTERIA AGAINST FISH PATHOGENS

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Abstract
The idea of this study is to investigate the antagonistic effect of *Sphingomonas* spp. against fish pathogens. The liquid culture inhibition test analysis showed that *Sphingomonas* spp. has an antagonistic effect on *Lactococcus garvieae* SY-LG1. However, the effect has not been fully observed on *Aeromonas salmonicida* subsp. *salmonicida* ATCC 33658, *Yersinia ruckeri* E42, *Vibrio (Listonella) anguillarum* and *Vibrio crassostreae* SY-VC10. In this study, the effect of *Sphingomonas* spp. on fish pathogenic bacteria was investigated for the first time through a liquid culture inhibition test and it was found that it had an antagonistic effect on *Lactococcus garvieae* SY-LG1. However, further studies are still needed for the definitive species diagnosis of *Sphingomonas* spp. and further tests such as enzyme production ability, pH resistance, hydrophobicity, and bile tolerance of the candidate probiotic isolates are encouraged. At the end of all these tests, it is also needed to investigate the resistance of fish against *L. garvieae* pathogen through dietary incorporation of *Sphingomonas* spp. in fish feeds.

Keywords: Fish disease, Liquid culture inhibition, Environment friendly substances, Antagonism

Introduction
Synthetic chemicals are currently used at different stages of production in both living and non-living products. According to the studies in the literature, we see that environmentally friendly substances can be used to replace synthetic chemicals in production. For example, copious

studies have been conducted on the effects of environmentally friendly products such as probiotics, prebiotics, plant extracts, organic acids, etc., to be used as an alternative to antibiotics and synthetic chemicals in fish farming within the last ten years (Hoseinifar et al., 2013). As a result, alternative additives and products are needed for healthy and environment friendly aquaculture. The advantages of probiotics include increasing the activity of digestive enzymes, activating immunity, inhibiting pathogens, increasing growth and survival rate, improving water quality, and biodegradation in organic sludge at the bottom of lakes (Balcázar et al., 2006; Nimrat et al., 2008; Cerezuela et al., 2011; Utiswannakul et al., 2011; Nimrat et al., 2012). Photosynthetic bacteria, yeasts, and *Pseudomona*s bacteria, and especially *Bacillus* and *Lactobacillus* bacteria are the most important probiotics used in aquaculture (Yilmaz et al., 2022; Abdel-Latif et al., 2022).

Studies conducted on the fish report that probiotic bacteria regulate intestinal flora and increase growth performance and immunity (Wang et al., 2008; Nayak, 2010; Mohapatra et al., 2013; Yilmaz et al., 2022; Abdel-Latif et al., 2022). Furthermore, many studies have proven the antagonistic effect of probiotic bacteria on fish pathogens (Van Doan et al., 2021). However, to the best knowledge of the researchers, there is no study conducted on the usability of *Sphingomonas* spp. as a probiotic in fish.

It is important to investigate the antagonistic effect of bacteria having probiotic potential on fish pathogens under *in vitr*o conditions. In this way, the effective pathogen on bacteria with probiotic potential can be determined before setting *in vivo* experiments on fish. The present study aims to investigate the antagonistic effect of *Sphingomonas* spp. having probiotic potential on pathogenic bacteria isolates that cause frequent disease in farmed fish.

Material and Method

Obtaining antimicrobial supernatant from probiotic bacteria

Methods reported in the literature were put into use to obtain antimicrobial supernatant from probiotic bacteria (Touraki et al., 2012; Balcázar & Rojas-Luna, 2007; Kongnum & Hongpattarakere, 2012). To this end, *Sphingomonas* spp. (previously isolated from rainbow trout intestine) culture was incubated in a 500 mL Erlenmeyer flask containing 250 mL of Tryptic Soy Broth (TSB) broth at 30 °C for 48 hours. Then, the broths containing the *Sphingomonas* spp. were centrifuged at 2142 x g for 20 minutes (Nüve NF 400) with an openable rotor in sterile falcon tubes and the precipitation of the bacterial colonies was ensured. After the precipitation, the supernatant containing the antimicrobial components were carefully taken and sterilized through a 0.45-µm millipore filter and then a 0.22 µm millipore filter. The antimicrobial effects of the *Sphingomonas* spp. supernatant on pathogenic bacteria were investigated by pH neutralization. As reported in the literature, 5N sodium hydroxide (NaOH) was used for pH neutralization and the pH of the supernatant was adjusted to 6.8 (Kongnum & Hongpattarakere, 2012).

Liquid culture inhibition test

For the liquid culture inhibition test, standard methods performed in 96 well plates as reported in the literature were used (Kongnum & Hongpattarakere, 2012; Nakayama et al., 2009; Khouiti & Simon, 1997). For the test, fish pathogens were produced in temperatures and broths as resented in Table 1; the intensity was set as 1 X 10^6 CFU/mL (Kongnum & Hongpattarakere, 2012). Subsequently, *Sphingomonas* spp. supernatants of 40 µl were placed on sterile plates and 160 µl broths containing fish pathogens were added to them. Then, the values were read at 600 nm wavelength. The plates were incubated at the appropriate temperature for the reproduction of the pathogenic bacteria. The values were read hourly on the microplate reader.

The antagonistic effect of the *Sphingomonas* spp. supernatant on fish pathogens was measured according to the graphs drawn with the reading results.

Table 1. Characteristics and culture conditions of the bacterial strains used in the study

Organism	**Description**	**Accession Numbers**	**Media (AMA/GRW)**	**Temp. (°C)**
Vibrio crassostreae SY-VC10	Fish pathogen / *D. labrax*	MG557819	MH*/TS*	24
Lactococcus garvieae SY-LG1	Fish pathogen / *O. mykiss*	KY118086	MH/TS	24
Vibrio (*Listonella*) *anguillarum*, SY-L24	Fish pathogen / *Dicentrarchus labrax*	KX388236	MH*/TS*	24
Yersinia ruckeri E42	Fish pathogen / *O. mykiss*	KX388238	MH/TS	22
Aeromonas salmonicida subsp. *salmonicida* ATCC 33658	Fish pathogen, collection strain	-	MH/TS	22

MH: Mueller-Hinton media; TS: tryptic soy media. *1.5% NaCl added to the media.

Results

The liquid culture inhibition test analysis showed that *Sphingomonas* spp. has an antagonistic effect on *Lactococcus garvieae* SY-LG1 (Figure 1). However, the effect has not been fully observed on *Aeromonas salmonicida* subsp. *salmonicida* ATCC 33658 (Figure 2), *Yersinia ruckeri E42* (Figure 3), *Vibrio (Listonella) anguillarum* (Figure 4) and *Vibrio crassostreae* SY-VC10 (Figure 5).

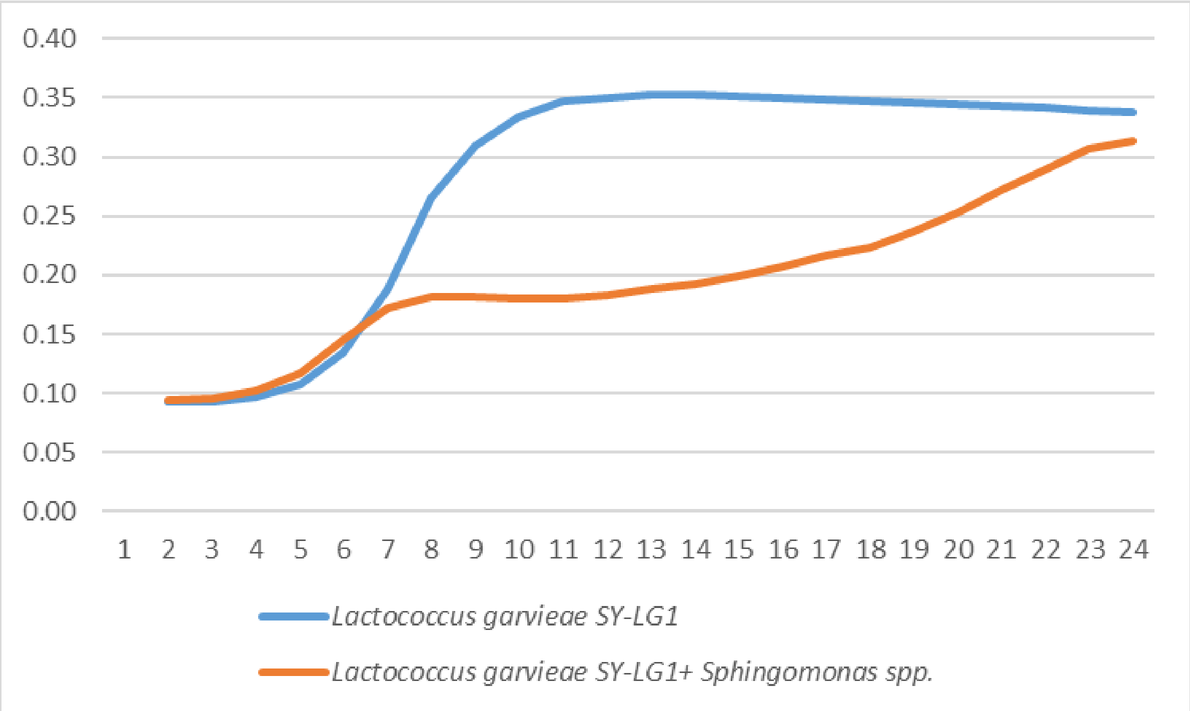

Figure 1. The antagonistic effect of the *Sphingomonas* spp. supernatant on the *Lactococcus garvieae* SY-LG1

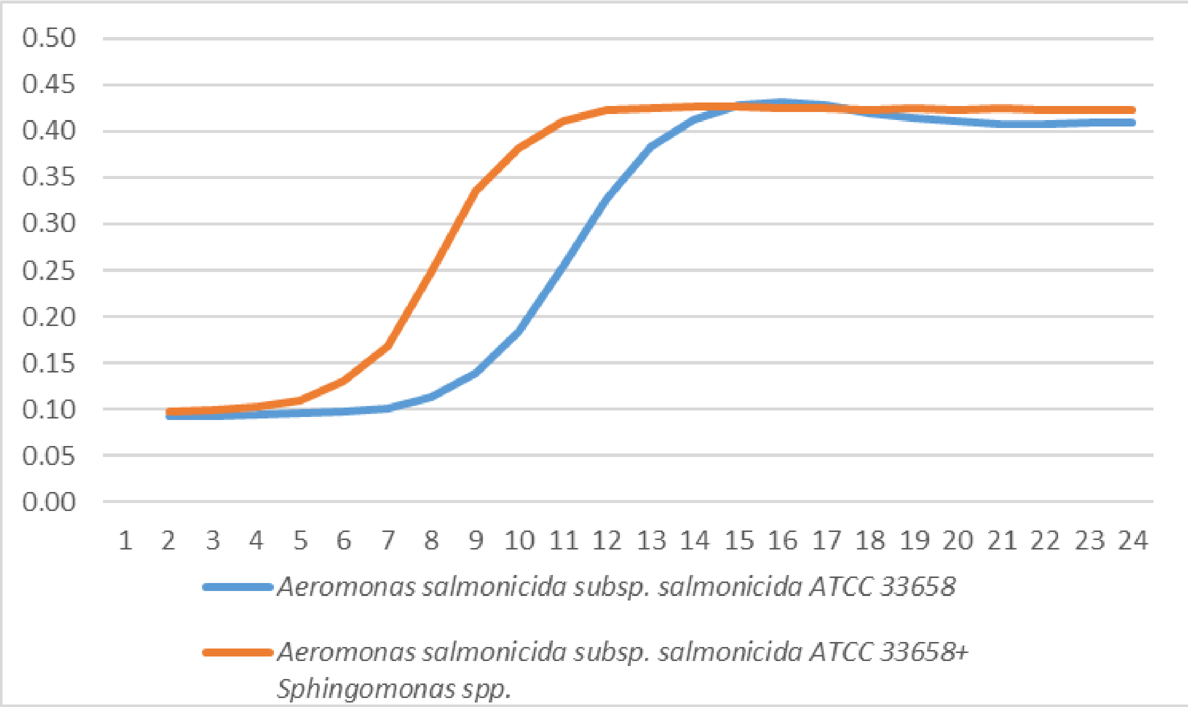

Figure 2. The antagonistic effect of the *Sphingomonas* spp. supernatant on the *Aeromonas salmonicida subsp. salmonicida* ATCC 33658

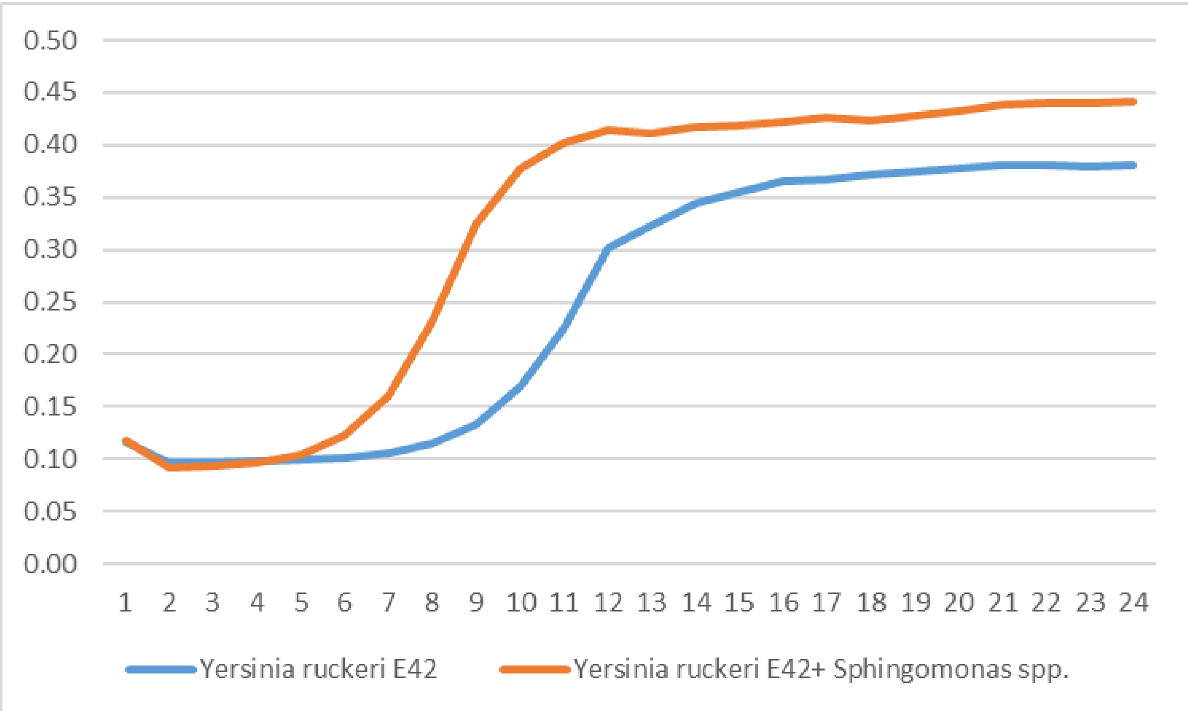

Figure 3. The antagonistic effect of the *Sphingomonas* spp. supernatant on the *Yersinia ruckeri E42*

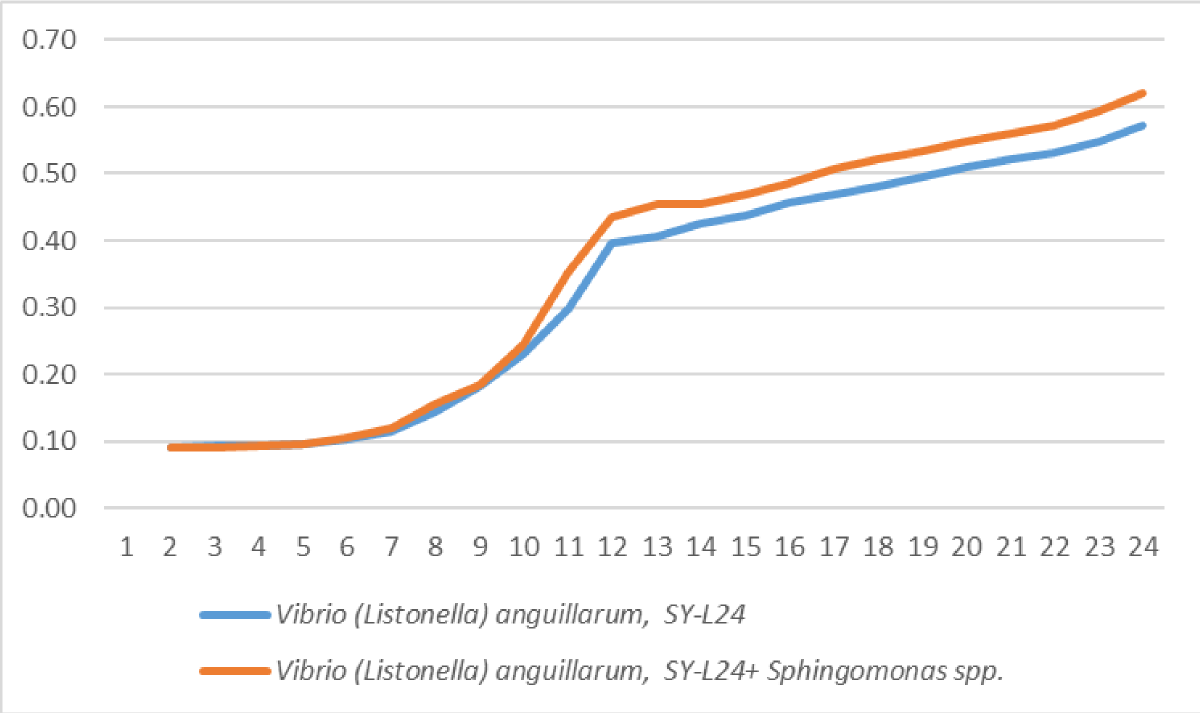

Figure 4. The antagonistic effect of the *Sphingomonas* spp. supernatant on the *Vibrio (Listonella) anguillarum, SY-L24*

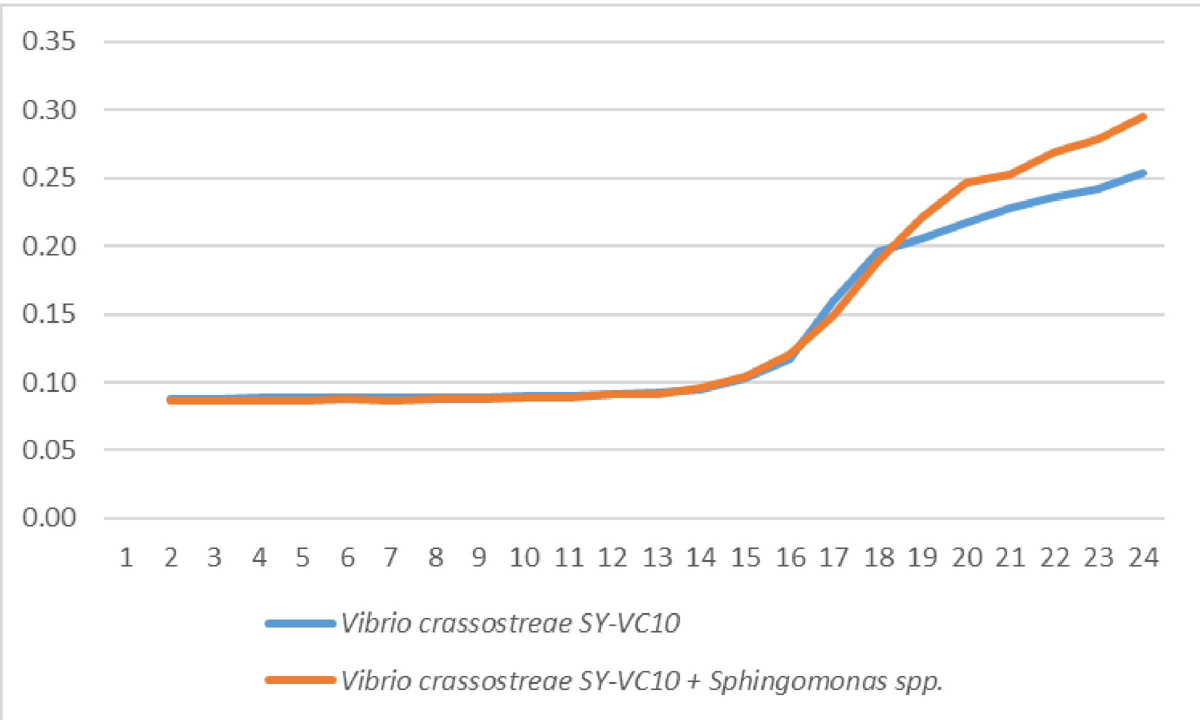

Figure 5. The antagonistic effect of the *Sphingomonas* spp. supernatant on the *Vibrio crassostreae SY-VC10*

Discussion

In this study, it was found that the *Sphingomonas* spp. supernatant has an antagonistic effect on *Lactococcus garvieae* SY-LG1. However, no study has been found in the literature on the antagonistic effects of *Sphingomonas* on fish pathogens. Therefore, the antagonistic effects of different probiotic bacteria on the fish pathogens used in this study are included in the discussion section.

Çelik et al. (2019) reported that *Bacillus subtilis* (ATCC 6633) supernatant mostly inhibits the growth of fish pathogenic bacteria as *A. sobria, E. tarda,* and *L. garvieae,* while *Lactobacillus plantarum* BC 7321 supernatant is mostly effective on the *A. sobria, L. garvieae, L. anguillarum,* and *Y. ruckeri*.

In another study, *Aeromonas sp.* A5, G1, and *Vibrio sp.* A12 isolates did not cause mortality on rainbow trout and showed probiotic properties with various tests, and it was reported that they showed an *in vitro* antagonistic effect against the pathogen *L. garvieae* (Didinen et al., 2014). Didinen et al., (2018) reported that *L. lactis* subsp. *lactis* M17/2-2 and *L. sakei* 2-3 isolates inhibited the development of *L. garvieae* and *V. salmoninarum* pathogens. Similarly, antagonistic effects of five different strains of *Lactobacillus acidophilus* on the *L. garvieae* were reported (Pehlivan & Onuk, 2020).

It was also reported that *Lactobacillus fermentum 24c, Pediococcus pentosaceus 10/9к, Lactobacillus paracasei 9c* probiotic bacteria isolated from carp fish did not have an antagonistic effect on *L. garvieae in vitro*, but the probiotic mixture added to the feed significantly increased the *L. garviea*e resistance of the fish (Tekebayeva et al., 2021). Different *in vivo* studies, on the other hand, reported the efficiency of *Aeromonas sobria* GC2 (Brunt & Austin, 2005), *Bacillus* spp. *JB-1* and A. *sobria* GC2 (Brunt et al., 2007), *L. plantarum* CLFP 238 *and L. mesenteroides* CLFP 196 (Vendrell et al., 2008)*, L. plantarum* subsp. *plantarum* CLFP 3*, L. lactis* subsp. *cremoris* CLFP 25 and *L. mesenteroides* CLFP 68 (Pérez-Sánchez et al., 2011) among probiotic bacteria isolates added to the trout feed in decreasing the mortality against *L. garvieae*.

Conclusion

In this study, the effect of *Sphingomonas* spp. on fish pathogenic bacteria was investigated for the first time through a liquid culture inhibition test and it was found that it had an antagonistic effect on *Lactococcus garvieae* SY-LG1. However, further studies are still needed for the definitive species diagnosis of *Sphingomonas* spp. and further tests such as enzyme production ability, pH resistance, hydrophobicity, and bile tolerance of the candidate probiotic isolate are encouraged. Overall, it is also suggested to investigate the resistance of fish against *L. garvieae* pathogen via dietary incorporation of *Sphingomonas* spp. in economically important fish species.

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Ethical approval

For this type of study, formal consent is not required.

Informed consent

Not available

Data availability statement

The authors declare that data are available from authors upon reasonable request.

Conflicts of interest

The authors declare no conflict of interest.

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Contribution of authors

Hüseyin ÇAKAR : Formal analysis, Writing original draft

Sevdan YILMAZ: Project administration, Conceptualization, Data curation, Investigation, Methodology, Writing original draft

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