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Investigation of *B. subtilis* viability at different pH ranges for use in microbial cleaner formulation

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Abstract: Until today, several disinfecting methods have been proposed and studied for cleaning and disinfection, containing heavy metals and chlorine, to reduce the biological load on environmental surfaces. Most of the proposed techniques are based on the use of chemical compounds. These methods have proven to be effective in reducing the majority of pathogens, but have been ineffective in preventing the persistence of pollutant microorganisms. Nowadays, increasing demand for natural and green products has led to recognition of cleaning products containing microbial based cultures. Cleaning products containing live microorganisms as active substances are becoming increasingly common in homes and industrial cleaning applications. These products are called "probiotic or microbial" cleaners. Microbial based cleaning products are environmentally cleaning products that contain bacteria or spores that are suitable as active ingredients. The purpose of using these cleaning systems is to prevent the development of pathogens by tolerating the presence of harmless probiotic microorganisms on surfaces. The aim of this study is to investigate the viability of *B. subtilis* species at different pH values for use in detergent formulation and the effective removal of pathogenic bacteria and fungi on the surface in the long term. As a result, it was observed that the probiotic *B.subtilis*, which can be used in the microbial based cleaner formulation, showed higher growth and vitality at the neutral pH and exhibited high antibiotic resistance, auto-aggregation, antagonistic and antifungal ability. Based on these findings, *B. subtilis* can be used to prevent the development of pathogenic species.

Keywords: *B. subtilis*; Probiotics; Microbial-based cleaning; Probiotic cleaning system

1 Introduction

Increasing demand for natural and green products has led to recognition of cleaning products containing microbial based cultures. Towards the early 2000s, new generation of microorganism-based products began to emerge for cleaning surfaces (Teasdale and Kademi 2018). Cleaning products containing live microorganisms as active substances have become increasingly common in houses and industrial cleaning applications (Spöka et al. 2018). Today, new generation cleaning products containing active microorganisms or spores as active ingredients attract the attention of professional users, consumer organizations and regulators. These products are also called as "biological" cleaners, "probiotic" cleaners or "microbial" cleaners (Teasdale and Kademi 2018; Spöka et al. 2018). In some studies, microbial cleaning products have proven to be effective in preventing contamination of surfaces with various pathogens. In addition, microbial cleaning products have been shown to reduce the presence of pathogens by 80-90% compared to the microbial load detected on surfaces treated with conventional cleaner / disinfectants (Brooke and Selby 2014; Vandini et al. 2014; Caselli et al. 2016; Caselli 2017; D'Accolti 2018; Caselli et al. 2019). The purpose of these microbial cleaning systems is to prevent the development of pathogenic species by tolerating the presence of microorganisms that do not harm for humans, not by a general disinfection that minimizes the presence of microorganisms (Vandini et al. 2014). In other words, instead of removing microorganisms from the environment, it may bring a new understanding of cleaning systems aiming to establish a controlled and less harmful microbiota with microbial cleaning products (Vandini et al. 2014; Caselli 2017). This approach considers that replacing all pathogens with beneficial microbes may be more effective at reducing infections (Caselli 2017). It is generally accepted that beneficial microbes are important for our health and their use can be effective in the prevention and treatment of infectious diseases. Among the potentially beneficial microorganisms for this purpose, "probiotics" are particularly preferred because they are defined as useful microorganisms for health (Vandini et al. 2014; Caselli 2017). Probiotics are beneficial microorganisms that benefit host health when taken in sufficient quantities (Gómez et al. 2016). Because of the therapeutic properties of probiotics, they have been used as food for many years. Probiotics are investigated as their survival in stomach conditions, colonization in the gut, antimicrobial effects against foodborne pathogens and other

functional properties (Lee et al. 2013). New generation cleaning products contain beneficial microorganisms that can compete with pathogenic microorganisms using nutrients on contaminated surfaces. These beneficial microorganisms are preferred because they remain on the surface where they are applied (often spores; bacteria that form spores in many formulations, *Bacillus* spp.) and prevent colonization by pathogenic microorganisms (Spöka et al. 2018). *Bacillus* is a genus of Gram-positive nonpathogenic bacteria, found in many places in nature (such as in soil, water, vegetables, human intestine) and have safe use in humans (Caselli 2017). *Bacillus clausii*, *Bacillus subtilis*, *Bacillus pumilus,* and *Bacillus coagulans* are specific species of the *Bacillus* genus that used as probiotics (Hong et al. 2008). Microbial cleaning products are more susceptible to microbial contamination and degradation due to their mild physical and chemical properties than conventional chemical cleaners with extreme pH and hard components. Therefore, all steps to be performed after fermentation should be carried out carefully to minimize the presence of external microorganisms contaminated detergent (Teasdale and Kademi 2018; Spöka et al. 2018). If storage conditions are not optimal, this cause different levels of stress for microbial components in the products and can cause a decrease in their viability despite the high resistance of *Bacillus* spores (Teasdale and Kademi 2018). In addition to storage and transport conditions, pH, the quality of spores and the biocompatibility of formulation components as well as the nature and concentration of preservative used are factors that can contribute to reducing the stability of *Bacillus* (Teasdale and Kademi 2018; Spöka et al. 2018). pH plays an important role in the stability and maintenance of the vitality of *Bacillus* spores. The optimal stability of *Bacillus* spores was found at pH 6.8 in a liquid medium and pH below 4.8 had a significant effect in reducing their viability over time, in the Teasdale and Kademi's study. The quality of spores also showed an important effect on maintaining their vitality in the liquid environment (Teasdale and Kademi 2018). The aim of this study is to investigate the viability of *B. subtilis* species at different pH values for use in detergent formulation and to investigate the effective removal of pathogenic bacteria and fungi on the surface in the long term.

2 Materials and Method

2.1 Pathogenic test cultures

The bacterial test cultures of *K. pneumoniae* ATCC 43816 and *P. aeruginosa* ATCC 27853 and fungi of the *Aspergillus brasiliensis* ATCC 16404 species were obtained from the culture collection of Algal Biotechnology and Bioprocess Laboratory of Yıldız Technical University. Stock cultures of bacteria were stored at -20 °C in Nutrient agar (NA) medium, supplemented with 25 % (v/v) glycerol. Pathogenic bacteria was incubated for 20-24 h at 37 °C in Nutrient agar (NA) medium (Vandini et al. 2014). Potato dextrose agar (PDA) was chosen as fungal medium for the growth of fungi and it was incubated at 28 °C for 6 days (Vehapi et al. 2018).

2.2 Cryopreservation of probiotic bacteria

Probiotic *Bacillus subtilis* ATCC 6633 was obtained from the culture collection of Algae Biotechnology and Bioprocess Laboratory of Yıldız Technical University. Probiotic strain *B. subtilis* was grown in 10 mL Luria Bertani (LB) medium at 37 °C for 24 h, and then incubated at 37 °C for 24 hours on Tryptic Soy Agar (TSA) plate. The cultures from the agar plate with the loop were transferred to eppendorf tubes that contain 500 µL of double LB and 500 µL of glycerol and stored as stock at -80 °C for future experiments.

2.3 Production of probiotic bacteria

B. subtilis ATCC 6633 stored at -80 °C, was activated by adding to the Luria Bertani (LB) liquid medium. Afterwards, planting was done on solid media containing Tryptic Soy Agar (TSA). Petri dishes were incubated overnight at 37 °C (Teasdale and Kademi 2018). Pure culture was obtained from the colonies grown in Petri dishes. For this, bacteria taken from a single colony were incubated in a 10 mL liquid medium (LB) at 35 °C and at different pH ranges such as pH 3, 5, 6, 7 and 8.

2.4 Investigation of growth kinetics

When the bacterium enters the liquid medium, a short lag phase, which passes to adapt to the medium, was observed. This was followed by the logarithmic phase in which the proliferation continues at a constant rate. As the nutrients in the environment were exhausted, the stationary phase began. By the way, some bacterial cells started to die (Winslow and Walker 1939). *B. subtilis* cell growth was monitored by measuring the optical density at 600 nm by taking samples from the culture grown in 10 mL LB medium hourly. PG Intruments T-60 UV Visible Spectrophotometer device was used for optical density analysis (Bhatta et al. 2006).

2.5 Calculation of plate count

Probiotic bacteria numeration was evaluated by pour plate method with serial dilution. Culture samples were serially diluted with phosphate buffered saline (PBS) according to the pour plate method, and 100 µL probiotic bacteria was spread on TSA plate, and incubated at 37 °C for 20-24 h. The number of Colony Forming Units (CFU) was determined by colony count after incubation of TSA plate. *Bacillus* colonies developing after incubation were counted and expressed in CFU mL^{-1} (Vandini et al. 2014; Bauzad et al. 2019). The numbers of bacteria per mL were calculated using the Eq. (1).

$$CFU\ mL^{-1} = (CFU \times DF) / aliquot \quad (1)$$

CFU: number of colonies (Colony Forming Unit)

DF: dilution factor

2.6 Auto-aggregation ability

Cell-cell interactions were evaluated according to the auto-aggregation test. Probiotic bacterial cells grown for 20 h at 35 °C in LB medium were collected by centrifugation. Optical density (OD 600nm) with phosphate buffered saline (PBS; pH 7.2) was adjust to A_0 (A_0=0.5). The number of bacteria (10^6-10^7 CFU mL^{-1}) was standardized in this way. Each bacterial suspension (8 mL) was vortexed and incubated at 30 and 37 °C for 7 h. Absorbance values were measured at 600 nm after 7 h (A_t) (Collado et al. 2008; Woo and Ahn 2013; Gómez et al. 2016). Auto-aggregation ability was calculated with Eq. (2).

Auto - aggregation (%) = $(1 - A_t / A_0) \times 100$ (2)

A_t: absorbance value measured at 7 th hour

A_0: absorbance value measured at initial time

2.7 Antibiotic susceptibility test

The antibiotic susceptibility test was performed by using the Bauer-Kirby method (Bauer et al. 1966). The Kirby-Bauer test, known as the disc diffusion method, is an antibiotic susceptibility test. For this test, a culture medium was properly and aseptically inoculated with the test organism, and then filter paper discs with a certain antibiotic concentration were placed in the medium. While the organism grows on the agar plate, the antibiotic inhibits growth. If the organism is sensitive to a specific antibiotic, no growth around the disc containing the antibiotic is observed. Therefore, an "inhibition zone" is measured to determine the susceptibility of microorganism to an antibiotic. It is then classified as microorganism Resistant (R), Medium (I), or Sensitive (S) (Bauer et al. 1966).

2.8 Antagonistic activity of *B. subtilis*

This test determines the antagonistic ability of the *B. subtilis* against pathogenic bacteria like as *K. pneumoniae* and *P. aeruginosa*. Antagonistic activity of *B. subtilis* against pathogens were studied by the method of antagonism in solid medium. The antagonistic activity was detected as a zone of pathogens' growth inhibition. *B. subtilis* grown in 10 mL LB medium for 30 h at 35 ºC were centrifuged at 12000 rpm for 15 minutes. Indicator pathogenic strains were subcultured on nutrient broth (NB) for 24 h at 37 °C. Nutrient agar (NA) plates were spread with 100 µL of indicator bacteria. *Bacillus* supernatant was dropped 30 µL into the wells (approximately 6 mm) on the surface of the nutrient agar (NA) plate containing pathogenic indicator strains at 10^8 CFU mL^{-1}, and the plates were incubated at 37 ºC for 24 h. Antagonistic activity was identified as the growth of *B. subtilis* around the well and the inhibition of the pathogen (Hong et al. 2008; Moore et al. 2013).

2.9 Antifungal activity of *B. subtilis*

In disc diffusion test, petri plates for pathogenic fungal microorganism "*Aspergillus brasiliensis* ATCC 16404" containing sterile Potato Dextrose Agar (PDA) growth medium (15 mL $plate^{-1}$), were inoculated with 6 mm diameter mycelium disc taken from 7 days old fungal cultures. Then, it was placed in the middle of the petri dishes (Vehapi et al. 2018, Özçimen 2018). *Bacillus* supernatant was dropped as 30 and 50 µL into the paper disc, and paper disc was placed on the cover of the petri dish. All the plates were inverted and incubated at a temperature of 28 °C for 6 days to allow adequate *Bacillus* - pathogen interaction to take place. All the cultured plates were periodically observed for mycelial growth. After incubation, fungal growth was measured and antifungal activity was evaluated by disc diffusion assay (Killani et al. 2011).

2.10 Statistical analysis

Data was presented as means with ± standard deviations. Analysis of variance was carried using the JMP (release 6.0.0, SAS) analysis program. The significance ratings between the averages were determined by Student's t test ($p < 0.05$ was considered significant).

3 Results and Discussion

3.1 Growth response of *B. subtilis* to variety pH

Growth curves of *B. subtilis* grown in media buffered at pH 3, 5, 6, 7 and 8 were shown in Figure 1. *B. subtilis* cells were incubated at 35 ºC for 30 h at various pH values from pH 3 to 8 in buffered LB nutrient medium and growth curves were obtained.

B. subtilis cultures in buffered modified medium of pH 7 and 8 were growth rapidly, whereas cultures of pH 5 and 6 showed a long lag time (Figure 1). A little difference was observed among the specific growth rates of *Bacillus* at pH 5, 6 and 8, indicating that there is a rather wide pH range for comparable growth of this bacteria under the conditions employed. As one would expect, the growth rate of the culture in the pH 3 medium rapidly decreased (Table 1).

In addition, it was observed that when the cells were grown at pH 3, 5 and 6, the adaptation phase lasted longer and some cells died after the adaptation phase. *B. subtilis* shows a lag in growth following an acidification, and the cells only at pH 3 switched to the death phase after the 6 th hour (Figure 1). Thus, growth in a acid induced adaptation to an acidic pH value such pH 5 and 6. In LB nutrient medium, the highest growth was observed at pH 7 and 8, and the lowest growth was at pH 3 and 35 ºC (Figure 1).

Specific cell growth rates and doubling time of *B. subtilis* that grown at different pH values were calculated using the OD600 values in the logarithmic phase of the growth curve of bacteria according to the Eqs. (3) - (4) (Table 1). The Monod equation is the most common rate expression to describe the growth of microorganisms (Wang et al., 2014; Baranyi and Pin, 1999; Ahmed et al., 2017). During the exponential phase we can write:

$dX/dt = \mu X$

$\ln(X/X_0) = \mu t$

$X = X_0 \times e^{\mu \Delta t}$ (3)

$t_{1/2} = \ln 2/\mu$ (4)

Where; t = time, X = CFU mL^{-1} at time t, X_0 = CFU mL^{-1} at time t_0, μ = specific growth rate constant (h^{-1}) and $t_{1/2}$=doubling time (h).

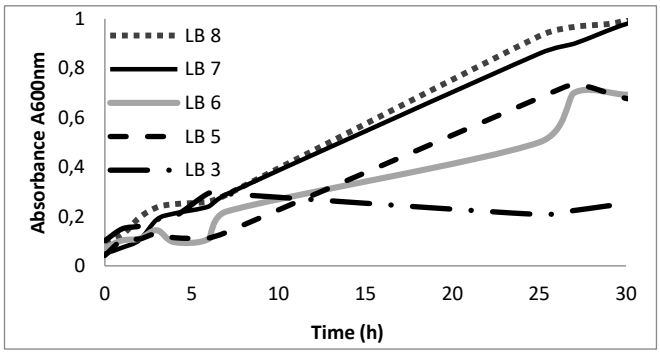

Fig. 1 Growth curves of *B. subtilis* grown in the LB medium buffered at pH 3, 5, 6, 7 and 8

Table 1 Specific growth rates and doubling time of *B. subtilis* at 35 °C in LB medium at different pH values

B. subtilis growth medium	Specific growth rate μ (h^{-1})	Doubling time $t_{1/2}$ (h)
pH 3	0.0125	55.2
pH 5	0.0703	9.81
pH 6	0.0756	9.12
pH 7	0.1112	6.20
pH 8	0.0782	8.82

Table 2 Pour plate count of *B. subtilis* probiotic strain

***Bacillus subtilis* ATCC 6633**							
Dilution	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	CFU mL^{-1}	Log CFU mL^{-1}
Count	TNTC	78	20	9	10	2.7×10^{6}	6.44

*TNTC: Too numerous to count

When the cells were grown at pH 3, 5 and 6, the adaptation phase was observed for the first 6 hours. After the adaptation phase, the growth phase was observed at pH 5, 6, 7 and 8, and the death phase was observed at pH 3 (Figure 1). The results showed that all pH values except pH 3 were suitable for cell growth (Figure 1), in addition, the specific cell growth rate at pH 7 was higher than pH 5, 6 and 8 (Table 1).

A similar lag phases in growth of *B. subtilis* was observed when cultures were shifted from pH 6 to pH 8.5 in the study of Wilks et al. 2009. Faille et al. 2002; suggested that when spores were suspended in acidic media, longer lag phases were observed. They compared pH 5 and 7 in their study and they observed better growth at pH 7 compared to pH 5. In addition, they observed the lag phase longer than 100 hours at pH 5 compared to pH 7.

3.2 Pour plate count of *B. subtilis*

The plate count method is commonly used in the pharmacy and food industry to determine the number of microorganism in a certain products. Probiotic based cleaners contain 10^6 CFU mL^{-1} spore probiotic bacteria (Collado et al. 2008; Arcales and Alolod 2018; De Cesare et al. 2019). The counts were listed in the Table 2 and Figure 2.

3.3 Auto-aggregation ability

Probiotic bacteria must accumulate and obtain a sufficient mass to show their beneficial effects (Collado et al. 2008). Its ability to accumulate is directly related to the first binding of bacteria to biotic and abiotic surfaces. The proposed mechanisms for antimicrobial activities of probiotic species include the production of compounds that prevent competition and pathogenic growth with pathogens for adhesion sites and nutrients and competitive exclusion of pathogens (Woo and Ahn 2013). Auto-aggregation ability, which is responsible for the ability to bind between cells, varies with temperature, osmolality and acidic conditions (Woo and Ahn 2013). The auto-aggregation ability of bacterial cells varied according to the incubation temperature. The highest auto-aggregation abilities were observed for *B. subtilis* (69.14%) incubated at 30 ºC in LB medium. However, lower auto-aggregation abilities of *B. subtilis* (65.50%) were observed at 37 ºC of LB medium. As a result, the auto-aggregation ability to collect probiotics is a desirable feature and varies depending on the temperature. Ritter et al. reported auto aggregation as 52.9% in their study with probiotic *Bacillus* isolates (Ritter et al., 2018).

3.4 Antibiotic susceptibility test results

Microbial resistance to antimicrobial agents is due to intrinsic properties of bacteria. Such intrinsic properties could make the bacteria capable of rapid inactivation of antibiotics through, exportation of the antibiotics out of the cell, or alteration of the specific antibiotic target site (Adimpong et al. 2012). The antimicrobial ability was determined against Chloramphenicol C30, Amoxicillin AML10, Ofloxacin OFX5, Fluconazole FCA25 (Table 3). Materials such as a sterile swab, forceps, drug and antimicrobial paper disk were prepared prior to the experiment and fresh 24 hours culture bacteria were used for the assay(Arcales and Alolod 2018). *B. subtilis* has been observed to be intermediate against Chloramphenicol C 30 μg, resistant to Amoxicillin AML 10 μg and Fluconazole FCA 25 μg and sensitive to Ofloxacin OFX 5 μg.

Table 3 Inhibition zone of *B. subtilis* against Chloramphenicol C30, Amoxicillin AML10, Ofloxacin OFX5, Fluconazole FCA25.

	Inhibition zone (mm)			
	C30	AML10	OFX5	FCA25
B.subtilis	15.5±0.70	0.00±0.00	22.5±0.70	0.00±0.00
	I	R	S	R

* Results are given as n = 3 ± standard deviation.

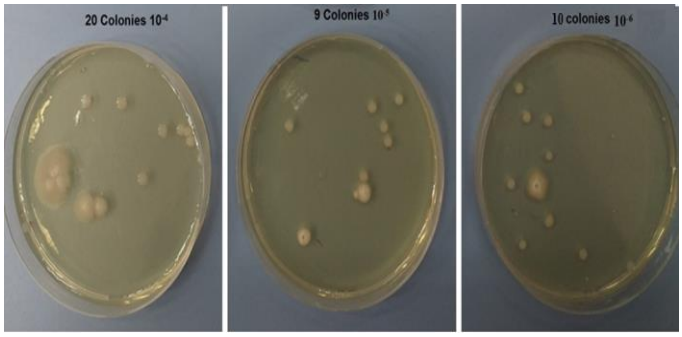

Fig. 2 Plate count of *B. subtilis* dilution with PBS.

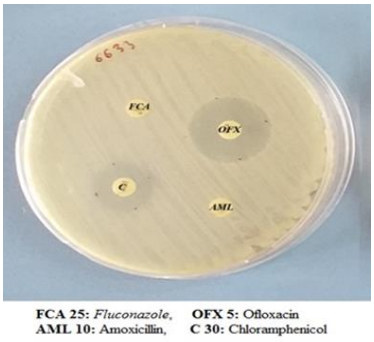

Fig. 3 Antibiotic resistance of *B. subtilis* to antimicrobial agents

Fluconazole FCA25 is used to prevent and treat various fungal and yeast infections. It acts by stopping the growth of certain fungal species (Aher et al. 2009). As seen in our study, Fluconazole FCA25 did not show any inhibitory properties against *Bacillus* species. As a result, it was observed that *B. subtilis* was more resistant to some antibiotics (Figure 3). Doganay and Aydin 1991; observed that the new antimicrobial agent, ofloxacin showed very good activity with MICs of 0.03 mg/L against *Bacillus anthracis* isolates. In our study, it was observed that ofloxacin with 5 µg concentration showed highest antibacterial activity with 22.50 mm inhibition zone against *B. subtilis*. Mazza et al. 1992; suggested that *B. subtilis* in the commercial probiotic product Enterogermina was resistant to chloramphenicol, tetracycline and streptomycin. In our study, it was observed that, *B. subtilis* ATCC 6633 was intermediate susceptible to chloramphenicol 30 µg with 15.50 mm inhibition zone. Weber et al. 1988; observed that the antibiotics such as penicillin, ampicillin, methicillin and cephalothin were highly active against *B. subtilis*.

3.5 Antagonistic activity of *B. subtilis*

Within the soil environment, probiotic bacteria compete for nutrients by the production of antibiotics that serve to inhibit the growth of their competitors (pathogenic bacteria). Indeed, the most of antibacterial compounds are natural products of soil-dwelling microorganisms (Hachmann et al. 2009). It was observed that the supernatant of *B. subtilis*, produced in 10 mL LB medium at 35 ºC for 30 hours at pH 7, inhibits *K. pneumoniae* and *P. aeruginosa* species (Figure 4).

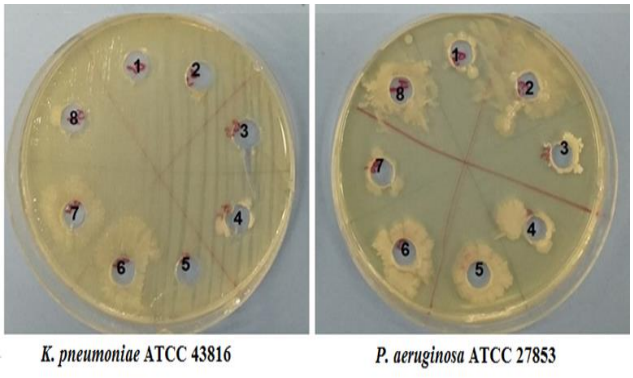

Fig. 4 Antagonistic activity of *B. subtilis* against *K. pneumoniae* and *P. Aeruginosa*

In this study, *B. subtilis* was growth at 35 ºC for 30 h at pH 7, after that, it was centrifuged and its supernatant was used as an antimicrobial agent against pathogenic species. As a result, *B. subtilis* inhibited pathogenic *K. pneumoniae* and *P. aeruginosa* strains. According to Figure 4, it is clear that *B. subtilis* has a higher antigonistic effect against *P. aeruginosa* than *K. pneumoniae*.

3.6 Antifungal activity of *B. subtilis*

Bacillus spp. are used for various biotechnological applications, such as probiotic cleaner, due to their ability to produce antimicrobial compounds inhibitory to pathogenic microorganisms in the surface enviroment (Adimpong et al. 2012).

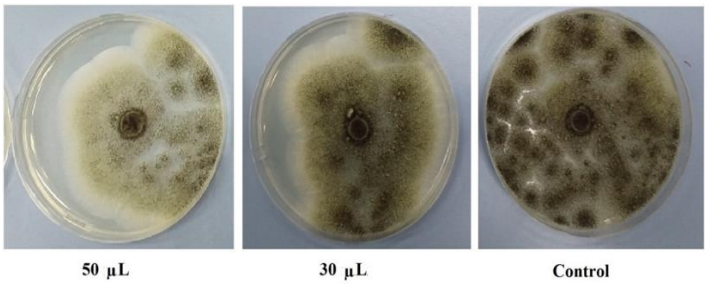

Fig. 5 Antifungal activity of *B. subtilis* at 30 and 50 µL doses against *Aspergillus brasiliensis* at 6 day of incubation

B. subtilis was growth at 35 ºC for 30 hours at pH 7 on LB medium, after that, it was centrifuged and its supernatant was used as an antifungal agent against *Aspergillus brasiliensis* ATCC 16404 mycelial growth. Increasing the doses dropped onto paper discs (30 and 50 µL) of *B.subtilis* supernatant resulted in weaker growth of fungi (Figure 5).

Table 4 Inhibitory effects of *B. subtilis* at different doses and in vitro mycelial growth levels of *A. brasiliensis*
Data are presented as means ± standard deviations (n=3).

	Mycelial growth diameter (mm)		
Incubation period	50 µL	30 µL	Control
3 day	$68.5\pm0.70^{A,a}$	$70.0\pm0.00^{B,a}$	$80.0\pm0.00^{C,a}$
4 day	$73.0\pm0.00^{A,b}$	$74.5\pm0.00^{B,b}$	$90.0\pm0.00^{C,b}$
5 day	$73.0\pm0.00^{A,b}$	$74.5\pm0.00^{B,b}$	$90.0\pm0.00^{C,b}$
6 day	$73.0\pm0.00^{A,b}$	$74.5\pm0.00^{B,b}$	$90.0\pm0.00^{C,b}$

a-b: Within each column, different superscript lowercase letters show differences between the *Bacillus* dose within each storage period ($p < 0.05$).

A-C: Within each row, different superscript uppercase letters show differences between the *Bacillus* dose and control ($p < 0.05$).

In-vitro effect of *B. subtilis* on fungal pathogen *Aspergillus brasiliensis* after 3 days showed average diameter of 68.50 - 70.00 mm of the mycelia of the fungal pathogen. In addition, the mycelia of the control fungal pathogen was observed as average 80.00 mm diameter. Results of fumigation application via *B. subtilis* supernatant, which were determined using the variance analysis JMP package program, showed that 50 µL dose of *B. subtilis* had the highest inhibition rates with 73.00 mm, compared with 30 µL dose with 74.5 mm inhibition rate and the the mycelia of the control fungal pathogen was observed average 90.00 mm diameter at 6 day of incubation (Table 4). As a result, *B. subtilis* used in this study successfully inhibited the growth of *Aspergillus brasiliensis*.

5 Conclusion

As a result, it was observed that the probiotic species "*B. subtilis*", which can be used in the detergent formulation, shows higher growth and vitality at the neutral pH value, while it passes into the death phase at acidic pH value such as pH 3. Therefore, it should be noted that surfactants and other additives to be used in the probiotic detergent formulation do not prevent the development of *B. subtilis* and do not lower the pH of the medium. In addition, *B. subtilis* has been observed to exhibit high antibiotic resistance and oto-aggregation ability.

According to antagonistic assay results, *B. subtilis* inhibited growth of indicator strain *K. pneumoniae* and *P. aeruginosa*. In addition, it can be derived from the Table 4 that *B.subtilis* exhibited high inhibitory effects on the growth of *Aspergillus brasiliensis* and the level of antifungal action was proportional to the doses (30µl, 50µl) that pipette onto paper discs. Results of this study corroborated that *B. subtilis* will effectively remove pathogenic bacteria and fungi on the surfaces, and the cleaner must contain the probiotic *Bacillus* species in order to effectively clean the pathogenic microorganisms on the surfaces in the long term.

Therefore, *B. subtilis* is a promising new strategic method in the probiotic/microbial cleaner formulation to remove pathogenic species from the environment and to prevent the development of pathogenic species in the long term.

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Authors' contributions: MV did the experiments of the growth of *B. subtilis* and antimicrobial tests. DÖ commented and reported all experimental results.

Conflict of interest disclosure:

There is no conflict of interest.

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Effect of different combinations of alpha lipoc acid, acitretin and methotrexate on malondialdehyde levels in the kidney tissues of rats

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Abstract: Acitretin (ACT), which is a second generation retinoid, exerts a therapeutic effect. It also has an anti-inflammatory and antiproliferative effect. It can also be used alone or in combination in the treatment of some diseases. In the use of methotrexate (MTX) alone, it can cause serious side effects such as hepotoxicity and nephrotoxicity. As a result of its use with ACT in recent years, it has been shown to have side effects on some organs as well as being beneficial. In this study, the effects of MTX, ACT and ALA on malondialdehyde (MDA) levels in rat kidney tissue were investigated. For this purpose, rats were given ALA, ACT + MTX and ACT + MTX + ALA by intraperitoneal injection. Following this, after the injections on the 3rd, 5th and 7th days, the kidneys were removed by applying cervical dislocation to the rats. The kidneys were homogenized, sonified and centrifuged. The fractions obtained as a result of these processes were used to investigate the effects on MDA levels. As a result, when compared to the MDA levels in the control group, an overall increase was observed in the group given MTX + ACT. It was determined that the increase continued with the administration of ALA with MTX + ACT. Only in the group where ALA was given, a decrease in MDA level was observed.

Keywords: Malondialdehyde; acitretin; methotrexate; alpha lipoic acid; kidney

1 Introduction

Acitretin (ACT), which is a seond generation retinoid monoaromatic, is a vitamin A analog (Khalil et al. 2017). It is also a retinoic acid metabolite of etretinate and is preferred due to its pharmacokinetic advantage (Özarmağan, 2016). ACT has antiproliferative, anti-inflammatory and immunomodulating properties (Tu et al. 2020). Besides being used in the treatment of diseases such as Hidradenitis suppurativa, Psoriasis, Pityriasis rubra pilaris, Lichen planus, Ichthyosis, Darier's disease, Actinic keratosis (Khalil et al. 2017), it also has a teratogenic effect since it is a retinoid (Özarmağan, 2016).

Methotrexate (MTX) belongs to an antineoplastic drug group, and is an analog of folic acid (Shingirik et al. 2019). It inhibits the conversion of dehydrofolate (DHF), which is necessary for the regulation of folic acid, to tetrahydrofolate (THF). Thus, it causes THF deficiency, which plays a role in the synthesis of purine and pyrimidine nucleotides, and causes disruptions in protein metabolism with nucleic acid (Güven et al. 2017). It is used for therapeutic purposes in diseases such as psyroatic and rheumatoid arthritis, psoriasis and ectopic pregnancy (Alinejad et al. 2019). At the same time, MTX is used in lung, breast, acute lymphoblastic leukemia, lymphoma, and head and neck cancers because it inhibits cell division (Güven et al. 2017). In addition to the therapeutic properties of MTX, toxic effects can also be seen. This toxicity occurs as a result of oxidative stress caused by free radicals (Ali et al. 2017). As a result of MTX-induced toxicity, the risk of intestinal-related sepsis, interstitial pneumonia in the lungs, and bone marrow suppression can be observed (Chabner et al. 2007). In addition, hepatoxicity and nephrotoxicity are also serious side effects of MTX (Şentürk, 2016). MTX, which causes toxicity even when used alone, has also been shown to have side effects in combination use. It is recommended to be careful in drug combinations against these side effects and to give an antioxidant together with combination use when necessary (Armağan, 2015).

Alpha lipoic acid **(**ALA) is an important naturally produced antioxidant that acts as a cofactor in mitochondial enzymes, found in most prokaryotic and eukaryotic cell types (Cadenas, 2001). ALA may have duties such as regeneration of vitamins C and E, the possibility of lipid peroxidation and protection against free radicals, increasing superoxide dismutase and catalase enzyme activities, repairing molecular damage and increasing acetylcholine production (Tetikçok et al. 2015). It has been observed that it regulates blood glucose in patients with Type 1 and Type 2 *Diabetes Mellitus* (Khamaisi et al. 1997) and helps healing in some diabetic retinopathic (Kilic et al. 1998) and neuropathic patiens (Ziegler et al. 1999). In addition, it has been observed that it has positive effects on cognitive functions in some neurological diseases (Cui et al. 2006). In addition to these, it also shows a protective effect against some

cardiovascular, neurological and diseases caused by viral infections (Özgün et al. 2018).

In light of this information, in recent years, although the side effects are known, the use of ACT + MTX combination has increased in the treatment (An et al. 2017). Therefore, in this study, the effect of ACT-MTX combination on malondialdehyde (MDA), which is a marker of cell damage in rat kidney tissue, and whether it has protective role of antioxidant ALA was investigated.

2 Materials and Method

2.1 Experimental Animals

In this experimental study, 50 Wistar albino type male rats (weighing between 250 and 300 g) were used all animals were obtained from the Ondokuz Mayıs University, Experimental Animals Application and Research Center (DEHAM), which was maintained for 12 hours in light and 12 hours in the dark and at a temperature of 21° C. The studies were approved by the Ethics Committee of Ondokuz Mayıs University (2018/ 13). The rats were fed with standard mouse food and water was given free.

2.2 Formation of Research Groups

The study groups were formed as the Control group (C), the ALA group, the ACT + MTX group and the ACT + MTX + ALA group. There are 15 rats in each of ALA, ACT + MTX, ACT + MTX + ALA groups that make up the experimental groups.

2.3 Injection and Obtaining Kidneys

Rats were starved for the last 24 hours before injection. Injection procedures were carried out at the same time every morning. ACT, MTX and ALA were resolved in 0.9 % NaCl, ACT (20 mg / kg / day) (An et al. 2017), MTX (20 mg / kg / week) (An et al. 2017), ALA (50 mg / kg / day) (Maritim et al. 2003) and their combinations were given to the rats as intraperitonal injection (i.p) at the body weight level of each. Rats in the experimental groups were sacrificed by cervical dislocation method by giving general anesthesia on the 3rd, 5th and 7th days after the injection. Rats were given 50 mg/kg ketamine HCl (ketalar) and 10 mg/kg Xylazine (Rompul) as general anesthesia. Following this, perfusion was performed with 0.9 % NaCl and kidneys were removed. These kidneys were placed in containers containing isotonic sucrose and stored in a freezer at -80 ° C for experimental procedures.

2.4 Preparation of Kidney Tissues for Analysis

After the tissues removed from the freezer were thawed, their weights were determined by weighing them on a sensitive scale. Then, homogenization, sonication and centrifugation processes were carried out according to the procedures. The supernatants obtained after centrifugation were stored at -80 ° C.

2.5 MDA Measurement in the Kidney Homogenates

MDA levels in kidney homogenates were measured by double warming, as described by Draper and Hadley. The principle of this method is the spectrophotometric measurement (at 532 nm) of the end product of the lipid peroxidation reaction with thiobarbituric acid (Draper and Hadley, 1990). An important marker of lipid peroxidation, MDA reacts with thiobarbituric acid (TBA) and forms a pink complex. The pink colored complex formation produced by MDA and thiobarbituric acid (TBA) reaction was evaluated by spectrophotometric measurement (at 532 nm). The MDA concentration was calculated with a calibration curve expressed as μmol / mg protein .

2.6 Statistics

All data were evaluated using SPSS 22.0 statistical package program. Since the groups did not show normal distribution, it was checked with Kruskal Wallis test whether there would be a difference between C, ALA, ACT + MTX and ACT + MTX + ALA groups. Values with $p < 0.05$ were considered significant.

3 Results

When the group given ALA is compared with the C group; On the 3rd day following the injection, a decrease of approximately 31% in MDA level was observed. This decrease was 28 % on the 5th day and 18% on the 7th day (Fig 1).

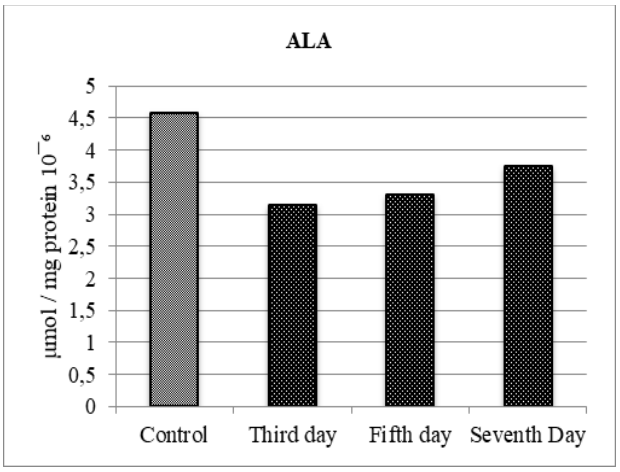

Fig.1 The levels of MDA in the ALA group

In the ACT + MTX group, the MDA level increased by 2 % on the 3rd day compared to the control. It was observed that the increase continued to increase on the 5th and 7th days and was approximately 10 % and 17 %, respectively (Fig 2). The ACT + MTX + ALA group increased by 98 % on the 3rd day compared to the control group. This increase, which was 16 % on the 5th day, was 31% compared to the control on the 7th day (Fig 3).

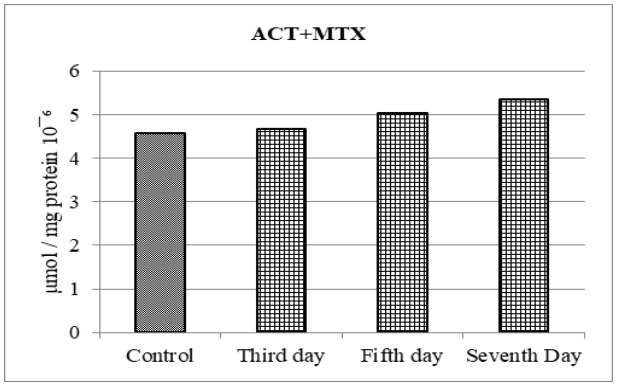

Fig. 2 The levels of MDA in the ACT+MTX

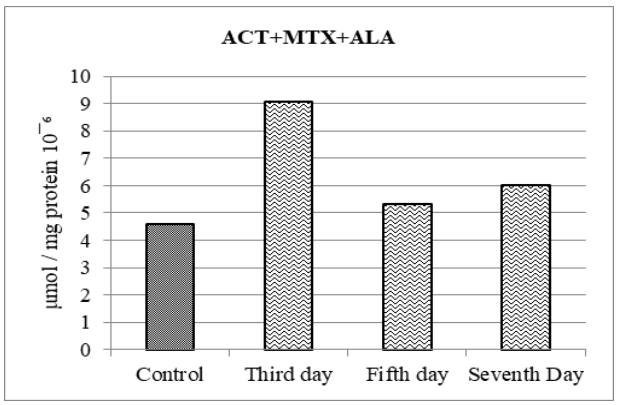

Fig. 3 The levels of MDA in the ACT+MTX+ALA

When we look at our work in general; compared to the C group, MDA levels decreased (p> 0.05) in the group given ALA and increased in the group given ACT+MTX (p>0.05). With the addition of ALA to the ACT+MTX combination , an increase in MDA levels was observed (p < 0,05) (Fig 4).

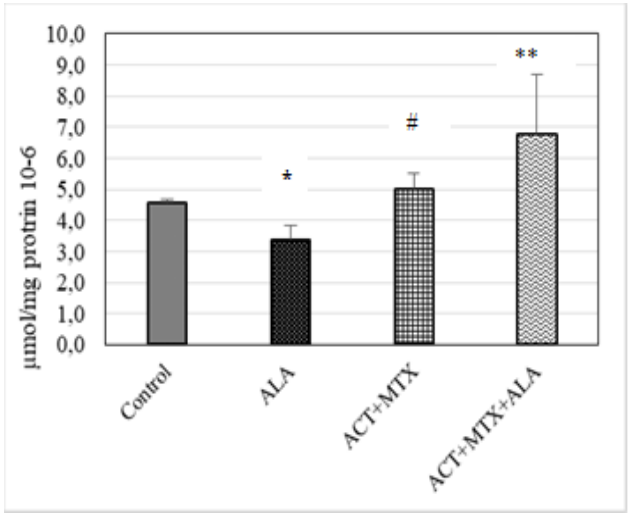

Fig. 4 The levels of MDA in the ALA, ACT+MTX and ACT+MTX+ALA groups

(*p> 0.05, ALA group compared to the control group)
(#p>0.05, ACT+MTX group compared to the control group)
(**p<0.05, ACT+MTX+ALA group compared to the control group)

4 Discussion

This study; It was made to investigate the effect that will occur as a result of the addition of an antioxidant ALA to the changes on MDA levels, which is an oxidative stress marker in rat kidney tissues that have applied ACT and MTX.

ACT is the secondary generation among systemic retinoids and is used for therapeutic purposes, especially in the field of dermatology (Khalil et al. 2017). However, in its use alone; Side effects such as an increase in transminase, triglyceride and cholesterol values and a decrease in HDL can be seen. Although these side effects are dose and time dependent, caution should be exercised and regular follow-up should be taken during the drug intake due to its teratogenic effect. In combination treatment, side effects and various risks can be seen while increasing effectiveness (Özarmağan, 2016).

MTX provides suppression of dermal inflammatory infiltration and prevention of cell proliferation. It is also used in the treatment of diseases in the field of dermatology and cancer. Also (Şentürk, 2016) in the compilation study; In addition to its therapeutic effect, it emphasized the necessity to pay particular attention to it single use and combined use as it has toxic properties on various tissues and organs such as hepatoxicity and nephrotoxicity. She also stated that dosage and duration adjustment should be done well and careful monitoring of patients against toxicity.

ALA can prevent the formation of reactive oxygen species as it forms chelates with metals. Thus, it has been determined that it can reduce mortality by using it as a treatment in some metal poisoning. In addition, it has been stated that it is a powerful antioxidant that can be used in the treatment of various diseases in order to stop and prevent the progression of tissues as a result of oxidative stress (Bludovska et al. 1999; Gomes and Negrato, 2014). In addition, no excessive side effects were found unless there was an overdose. It can be used to reduce and prevent the toxicity that may occur in combination therapies and the treatment of toxic drugs (Yürük and Ayaz, 2014; Ergene, 2018).

In one study, it has been concluded that ACT and MTX combination therapy are not faced with a new or extraordinary condition, including hepatitis or liver toxicity. And it has been reported that it can be used as a combination of ACT and MTX at low doses. It has also been stated that simultaneous use of MTX will not be an absolute contraindication in patients who will benefit from the use of ACT (Lowenthal et al. 2008).

An et al. (2017), when ACT and MTX combination groups are compared with MTX used groups they found that the increase in profibrotic factors in serum was less. In addition, in this study, they thought that ACT and MTX combination may provide higher efficacy in the treatment of psoriasis and decrease in liver fibrosis. As a result, it is stated that if appropriate doses are used, ACT and MTX can be used as a combination.

In our study, it was observed that in the groups in which ACT + MTX was given, it caused an increase in MDA level. This increase in MDA level is also known as a marker of damage to kidney tissue. It is thought that the reason for this situation may be due to the toxic effect of MTX.

In the study of Armağan et al. (2015), it was observed that there was an increase in MDA level in kidney tissu es of MTX group compared to C. It was observed that this increase decreased in the treatment group that received MTX + ALA. It has also been concluded that ALA is effective with antioxidant and other properties in preventing the toxic effect of MTX in kidney tissue.

In another study, the effects of luteolin (LUT) with strong antioxidant and anti-inflammatory properties against MTX-induced liver toxicity in rats were investigated. As a result of the researches, an increase in MDA level was observed in the groups that received MTX in liver tissue. It was observed that this increase decreased in MTX and LUT applied groups. As a result of the biochemical evaluations, it was observed that while MDA values increased in the group receiving MTX in liver tissue, it decreased in the LUT group. It has been concluded that LUT treatment reduces MTX-induced damage (Gedik, 2019).

In our study, while the increase of MDA level was observed in the group given ACT + MTX, it was also determined that it did not decrease in the ACT + MTX + ALA experimental group. Thus, it has been observed that ALA, which is used as an antioxidant, causes an increase in MDA in all the days it is added to the combination. However, when using ALA alone, it was seen that there was a decrease in MDA levels. And it has been determined that ALA does not have a condition that causes tissue damage when used alone.

Aksoy (2017) investigated the effect of ALA on testicle damage caused by busulfan (BUS) in his study. As a result of the study; Compared with the C group, it was observed that the MDA level was high in the group where BUS was given, it was less in the group that received ALA, and there was an increase in the group that received BUS + ALA. As a result, it was observed that BUS caused damage to the testicles, but it did not show its protective and / or therapeutic effect in the use of ALA with BUS.

When our results are compared with the literatüre, it was found to be in agreement with the results in testicular tissue while in contrast with the results in liver and kidney tissue.

5 Conclusion

As a result, it was observed that the combined use of ACT and MTX caused damage to rat kidney tissue. With the addition of an antioxidant ALA to this combination application, while the damage is expected to decrease, an increase in the indicator MDA has been observed. Thus, in this study, it was concluded that ALA did not show a therapeutic effect in use with ACT and MTX for kidney tissue.

According to our data , it was thought that a study to be conducted using an increase in the number of days and using different doses could contribute to the literature.

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Authors' contributions: B.B.,E.D.and F.G.S.designed and performed the experiment. They also analyzed the data and wrote the manuscript.

Conflict of interest disclosure:

We have no conflict of interest with any people or organization.

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Bulletin of Biotechnology

Determination of antimicrobial activity of different extracts of *Enteromorpha intestinalis* (Linnaeus Nees.1820) against pathogenic microorganisms

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Abstract: It is known that components such as polyphenols, flavonoids and polysaccharides have antimicrobial activity in macroalgae in aquatic environments. In this study, it was aimed to determine the antimicrobial activities of *Enteromorpha intestinalis* (L.) Nees methanol, 70% ethanol and water extracts against different pathogenic microorganisms, which are economically important marine macroalgae. According to Broth Microdilution Method; Antimicrobial activities of different extracts of the studied species against *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Staphylococcus aureus*, *Salmonella enteritidis*, *Sarcina lutea* and *Bacillus c*ereus standard bacterial strains and *Candida albicans* fungus strain were evaluated by determining the Minimum Inhibition Concentration (MIC).According to the findings, it was observed that the water extract of the studied species showed no antimicrobial activity against any test organism. The methanol extract of *E.intestinalis* was found to have antimicrobial activity against *P. aeruginosa*, *S. aureus*, *S. lutea*, *B. cereus* and *C. albicans* strain, while ethanol extract was observed against *P. aeruginosa*, *S. lutea* and *C. albicans* strains. The highest antibacterial effect was observed in *E.intestinalis* (L.) Nees methanol extract and the most effective strain was *Bacillus cereus* (0.390 mg/mL). Nowadays, algae are of great importance in terms of being very useful drug raw material, containing the basic components, being more effective and less toxic, as well as being models for drugs with original drug-like physiological activity. Since the data obtained with this study are intended for prospective application, it is thought that alternative drug applications will be provided as a basis for the studies aimed at reducing the use of commonly used antibiotic and antifungal agents against pathogenic microorganisms.

Keywords: *Enteromorpha intestinalis*; macroalgae; antimicrobial activity; pathogen microorganism**.**

1 Introduction

In recent years, algae are among the natural resources that people started to use for their own benefit (Yoldaş et al. 2003; Sevindik, 2020). Algae are important bioactive molecule sources used in human and animal nutrition. There are many types of antimicrobial compounds that play an important role in the natural defense of all living species in nature (Rauha et al. 2000; Mohammed et al., 2019).

Antibiotic, antiviral, anticancer, antifungal, antibacterial, anti-inflammatory effects, as well as hypocholestrolemic, enzyme inhibition and some other pharmacological effects of these molecules from microalgae and macroalgae have been observed in the last two decades. These natural products are not only used as pharmaceutical raw materials, but also as structural models in the production of synthetic molecules (Sevindik, 2018). Macroalgae have low calorie, high vitamin, mineral and fiber content, and because of that, they become an attractive material for researchers. *E. intestinalis* (L.) Nees. is macroalgae and can grow in all natural waters (Akköz et al. 2011) (Fig 1.)

The antimicrobial activity of macroalgae is due to chlorophyll derivatives, acrylic acid, terpenes, phenolic substances, halogenated aliphatic components and sulfur-containing heterocyclic components. Besides these components, antimicrobial activity is stated to be caused by some amino acids, fluorotannins, steroids, halogenated ketones and alkanes, cyclic polysulfides and fatty acids (Gupta et al. 2012).

The aim of the present study was to determine antimicrobial activity of different extracts of *E. intestinalis* against pathogenic microorganisms. Systematic of *E.intestinalis* that we use in our study is as given in Table 1.

Fig. 1 *Enteromorpha intestinalis* (Linnaeus) Nees 1820

Table 1 Systematic of *Enteromorpha intestinalis* (Linnaeus) Nees 1820 (AlgaeBase)

Species	Genus	Family	Ordo	Classis	Subphylum	Phylum	Subkingdom	Kingdom
E. intestinalis	Enteromorpha	Ulvaceae	Ulvales	Ulvophyceae	Chlorophytina	Chlorophyta	Viridiplantae	Plantae

2 Materials and Method

2.1 Algae Samples Collection

Samples of *E. intestinalis* seaweed was collected from a sampling as deep as 0-1bm, station in Demre, far 150 km from Antalya, The position of Demre is centered on, 36°13'42.30"N -29°56'23.31"E. *E intestinalis*, whose geographical information has been given above, was washed with the ambient waters in order to remove foreign substances first and brought to the laboratory environment in sterile polyethylene bags. Washed with distilled water in the hydrobiology laboratory to remove epiphytic creatures and necrotic particles on the samples.

In order to accelerate the drying process of the macroalgae which drained the water and after this process, the algae were placed in an oven set at 40ºC to prevent the phytochemical compounds from being damaged, and pre-drying was carried out by keeping it for 17 hours. Algae that were properly dried and ground with the help of hand homogenizer were kept airtight at room temperature until extraction.

2.2 Preparation of Algae Extracts

Algae collected from the field were cleaned and dried in the shade. Soxhlet extraction method (Thermal) was applied after grinding with the help of a mechanical grinder to obtain extracts of shade-dried algae samples.

Powdered samples (20 g) were extracted for 6 hours at 65 ° C in a soxhlet extraction method using 180 mL of methanol solvent. After the extraction phase was obtained, after drying the samples, extraction was carried out with other solvents (70% ethanol (at 75 °C) and water). The extracts obtained were filtered through Whatman No: 1 filter paper and then evaporated (until 1-2 mL thick) at 40 °C under reduced pressure and stored in vials at -20 ° C until analysis. (Kaufman et al., 1995). The amount of extracts, organic solvent type, drug amount and extraction efficiency are as shown in Table 2. The graph showing the yields of algae extracts is as shown in Fig 2.

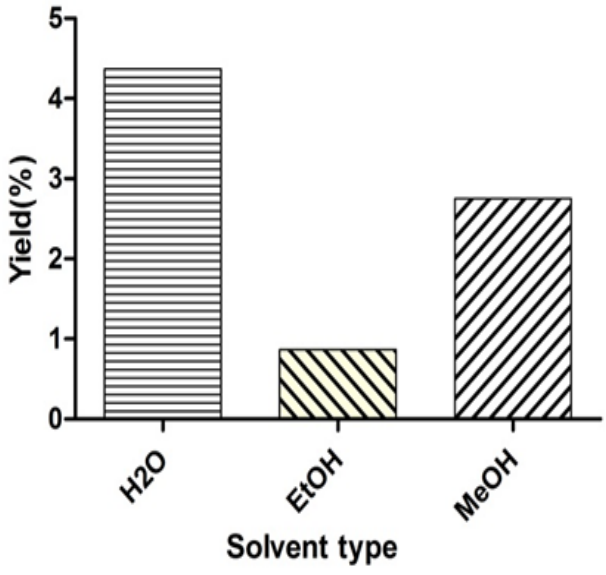

Fig. 2 The yields (%) of algae extracts.

When the extracts were to be tested for their antimicrobial activities, they were dissolved in DMSO at a concentration of 25 mg / mL for use in the broth microdilution method, and after being passed through 0.45 µm milipore filters, they were divided into small volumes in tubes with an extract number and stored at +4 ºC. (Buruk 2002).

Table 2 Amount of algae extracts, organic solvent type, drug amount and extraction yields.

Algae name	Collection area	Coordinate	Solvent type	Drog amount (gr)	Balloon joje tare(gr)	Last weighing (gr)	Extract amount	Yield %
E. intestinalis	Antalya Demre creek	**36°13'42.30"K 29°56'23.31"D**	**H_2O**	**20**	**69.91**	**70.78**	**0.87**	**4.37**
			EtOH	**20**	**112.28**	**112.45**	**0.17**	**0.87**
			MeOH	**20**	**113.89**	**114.44**	**0.55**	**2.75**

2.3 Preparation of Broth

To determine the antimicrobial activities of the extracts, some modifications were applied and the broth microdilution method reported by Abbasoğlu et al.(1995) was used (Abbasoğlu et al. 1995). Mueller-Hinton broth was used in this method. In addition, Brain Heart Infusion Broth broth was prepared to prepare fresh cultures of the test microorganisms overnight. After the Brain Heart Infusion Broth (BHIB) medium was homogeneously dissolved in distilled water with 37 g/L, it was distributed to 10-mL tubes with screw cap and sterilized in an autoclave at 121 ° C for 15 minutes. After the Mueller Hinton Broth medium (21 g / L) was dissolved in distilled water, it was dispensed into test tubes at 10 mL and sterilized in an autoclave at 121 ° C for 15 minutes.

2.4 Test microorganisms

The strains of three gram positive bacteria (*Staphylococcus aureus* ATCC 43300, *Bacillus cereus* ATTC 11778, *Sarcina lutea* ATTC 9341)and four gram negative bacteria (*Escherichia coli* ATTC 25922, *Pseudomonas aeruginosa* ATTC 27853, *Klebsiella pneumoniae* ATTC 70603, *Salmonella enteritidis* ATTC 13076) and also one strain of fungus (*Candida albicans*) were obtained from the culture collection of the Department of Biology Microbiology Laboratory of Selcuk University, Konya, Turkey, and were maintained on Brain Heart Infusion (BHI) agar medium at 4 ^{0}C until testing.

2.5 Antimicrobial testing

Minimum inhibitor concentration (MIC) tests were performed in accordance with the M27-A8 CLSI (Clinical Laboratory Standards Institute) criteria for bacteria (CLSI).Antimicrobial activity was evaluated using Broth Microdilution method. Briefly, the stock solution of the extract was obtained using solvent (Grierson and Afolayan, 1999) or DMSO (Salie et al. 1996; Nostro et al. 2000; Baris et al. 2006; Mendoza 1998).The inoculum volume for this procedure is usually 5 x 105 cfu / mL (Lourens et al. 2004; Basri and Fan 2005).

Microbial culture with 0.4 optical density at 620 nm or 12 hour broth culture set with 0.5 McFarland turbidity standard was used (Baris et al. 2006). 100 µL of the plates were added to the first wells of the extracts diluted with microbial culture and DMSO at concentrations of 25 mg/mL and incubated at 37 ° C for 24 hours (Lourens et al. 2004).

After incubation, plates were examined for changes in turbidity as an indicator of growth. The first well that appeared clear was considered the MIC of the extract. Dilutions of the extracts according to Log2 base (12.5 mg/ mL - 12.2 µg/mL) were prepared. No extract or culture was placed in the last well of the plate for media control. Algae extracts were diluted between concentrations of 6.25 mg / mL and 6.1 µg/mL. In parallel with this study, negative control (DMSO) and control antibiotic gentamicin were prepared in different plates. Reconstitution of gentamicin from 0.1 mg/ mL concentration was made with serial dilution at concentrations of 0.025 mg/mL-0.02 µg / mL. After 18 hours incubation at 37 °C, 20 µL of aqueous (aqueous) TTC (0.5%) was added to the wells and incubated for another 30 minutes at 37 °C. At the end of the incubation period, the growth in the plates was checked, and the lowest extract concentration in which there was no visible growth (uncolored areas) and hence inhibition of growth was evaluated as MIC**.**

3 Results

The antimicrobial activity results obtained in the broth microdilution method of *E. intestinalis* extracts are given in Table 2 and MIC of gentamisin(0.1mg/mL) and DMSO(%100) are demonstrated in Table 3.

Table 2. Antimicrobial activity of *E. İntestinalis* water, methanol and 70% ethanol extract

Test Microorganism	MIC (mg/mL)		
	***E.intestinalis* H_2O**	***E.intestinalis* Methanol**	***E.intestinalis* Ethanol(%70)**
A2	—	—	—
A8	—	**1.562 mg/mL** (3.well)	**1.562 mg/mL** (3.well)
A10	—	—	—
A11	—	**3.125 mg/mL** (2.well)	**3.125 mg/mL** (2.well)
A12	—	—	—
A14	—	**0.781 mg/mL** (4. well)	**1.562 mg/mL** (3. well)
A55	—	**0.390 mg/mL** (5.well)	**1.562 mg/mL** (3. well)
A17	—	**< 0.006 mg/mL**	**0.012 mg/mL** (10. well)

A2: *E. coli* ATCC 25922
A8: *P. aeruginosa* ATTC 27853
A10: *K. pneumoniae* ATCC 70603
A11: *S. aureus* ATTC 43300
A12: *S. enteritidis* ATTC 13076
A14: *S. lutea* ATTC 9341
A55: *B. cereus* ATTC 11778
A17: *C. albicans*
— *: There is bacterial growth*

Table 3. MIC of gentamisin(0.1mg/mL) and DMSO(%100)

Test Microorganism	Gentamisin (Positive control) (0.1 mg/mL)	DMSO (Negative control) (%100)
A2	**< 0.02 mg/mL**	**%12.5**
A8	**<0.02 mg/mL**	**%12.5**
A10	**0.78 mg/mL**	**%12.5**
A11	**<0.02 mg/mL**	**%25**
A12	**0.04 mg/mL**	**%12.5**
A14	**<0.02 mg/mL**	**%12.5**
A55	**<0.02 mg/mL**	**%12.5**
A17	**<0.02 mg/mL**	**%12.5**

4 Discussion

According to the findings, it was observed that the water extract of the studied species showed no antimicrobial activity against any test organism. The methanol extract of *E.intestinalis* was found to be effective in terms of antimicrobial activity against *P. aeruginosa*, *S. aureus* , *S. lutea* and *B. cereus* strains of bacteria and *Candida albicans* fungus strain. It was observed that *E.intestinalis* methanol and ethanol extract were effective only against *P. aeruginosa* strain from gram negative bacteria, while methanol extract did not show antimicrobial effects only against *S. enteritidis.* In other words, it can be said that the methanol extract of the species is more effective against gram positive bacterial strains.

Mansuya et al. (2010) studied antibacterial activity of *Cladophora glomerata* Grunow, *Ulva lactuca* L., *U. reticulata* Forsskål, *Gracilaria corticata (J.Agardh) J.Agardh, Kappaphycus alvarezii* (Doty) Doty ex P.C.Silva and *Sargassum wightii* Greville ex J.Agardh extracts by well diffusion method. Maximum activity (45 mm) was recorded against *U. reticulata* 200 mg of *Salmonella typhi* and minimum activity (9 mm) was recorded against *Streptococcus pyogenes*, while 50 mg of *U. lactuca* water extract was recorded and methanol extracts have been reported to have higher antimicrobial activity than water extracts (Manyusa et al. 2010)

Zbakh et al. (2012) tested the antibacterial activity of 20 macroalgae species (9 green algae, 3 brown algae and 8 red algae) collected from the Morocco coasts of the Mediterranean against *Escherichia coli*, *Staphylococcus aureus* and *Enterococcus faecalis.* Among algae tested, 17 were found to show antibacterial activity. Extracts of *U. lactuca*, *Gracilaria bursa-pastoris* (S.G.Gmelin) P.C.Silva and *Chaetomorpha linum* (O.F.Müller) Kützing were determined to have the highest antibacterial activity. Methanol extract of *Ulva rigida* C.Agardh has been reported to have an inhibitory effect against all strains tested (Zbakh et al. 2012).

Varier et al. (2013) determined the antimicrobial activity of red algae (*Gelidiella acerosa* (Forsskål) Feldmann & Hamel, *Gracilaria verrucosa* (Hudson) Papenfuss and *Hypnea musciformis (*Wulfen) J.V.Lamouroux) against gram positive and negative bacteria by disc diffusion method. Methanol, ethanol, chloroform and water were used as the solvent. While chloroform extracts of *G. verrucosa* were reported to constitute the highest inhibition zone (21 mm) against *Salmonella paratyphi*, none of the water extracts showed antibacterial activity (Varier et al. 2013).

5 Conclusion

As a result, it has been determined that extracts obtained using different solvents belonging to algae type used in our study have different degrees of antibacterial effects against the tested bacteria, and it will be appropriate to optimize the production conditions of the active substance, characterize it and to clarify the mechanism of action. It has been determined that the species used in the study has high antimicrobial activity, and it is suggested that different studies can be conducted in the future for the use of new drugs produced for the treatment of infectious diseases as antimicrobial agents. In the light of new studies, it will be possible to contribute to the development of herbal natural products that can be used against various diseases by pharmaceutical companies and relevant scientific authorities.

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The investigation of the effect of alpha lipoic acid on lung polyphenol oxidase activity in acitretin and methotrexate given rats

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Abstract: Oxidative stress is one of the main causes of lung damage caused by methotrexate (MTX). In addition, it has been observed that it causes serious side effects on other organs. In recent years, MTX has started to be used in therapy in combination with Acitretin (ACT). Alpha lipoic acid (ALA), which has antioxidant and anti-inflammatory activities, is naturally found in human foods. In this study, it was aimed to investigate the effect of ALA on phenol oxidase activity in lung tissue in the elimination of cellular level damage by free radicals produced by ACT and MTX. In the study, 50 Wistar albino male adult rats, selected from the same generation and weighing between 200 and 250 g, were used. The rats used were fed with standard mouse food and water was given free. Study groups were formed as control group (K), ALA group, ACT + MTX group and ACT + MTX + ALA group. ACT (20mg / kg / day), MTX (20mg / kg / week), ALA (50mg / kg / day) were dissolved in 0.9% NaCl and given to rats intraperitoneally. Polyphenol oxidase (PO) (1.10.3.1.) activation, which catalyzes the oxidation of phenolic compounds, was determined by the Hung and Boucias (1996) method. Compared to the control group, PO activity was found to be 66% higher in the MTX + ACT group and 46% higher in the MTX + ACT + ALA group. On day 5, the PO activity of the MTX + ACT + ALA group was 55% lower than in the MTX + ACT group, and this decrease was found statistically significant ($p < 0.05$). It is determined that this decrease has reduced to 20% on the 7th day. In the study, it was observed that the combined use of ACT and MTX increased phenol oxidase activity in rat lung tissue. A decrease in this activity was observed with the addition of an antioxidant ALA to this combined application.

Keywords: Alpha Lipoic Acid, Acitretin, Methotrexate, Phenol Oxidase

1 Introduction

Polyphenol oxidase (1,2-benzenediol:oxygen oxidareductase; EC 1.10.3.1) is an enzyme, which catalyzes the oxidation of phenolic compounds with molecular oxygen. These enzymes contain copper at their active site. This metal ion enables them to oxidize the phenolic group of an aromatic compound to a reaction group known as a Quinone (Whitaker 1995). This enzyme is found in many plant tissues, in some fungi, and in some higher animals, including insects and humans. (Wu et al. 2008). Polyphenol oxidase frequently called tyrosinase, phenolase, polyphenolase, catecol oxidase, cresolase or catecholase. The name *tyrosinase* came from tyrosine being used first as a substrate. It seems preferable to reserve the name tyrosinase for the mammalian enzyme, which has more limited specificity in that it acts only on tyrosine and dihydroxyphenilalanine. A number of important diseases in humans result from lack of sufficient activity or too much activity (Whitaker 1995). 4-hydroxyanisole a substrate of tyrosinase, was found to cause regression of Harding-Pasey melonama in mice. Another important area in which tyrosinase is used is the production of L-DOPA used in the treatment of Parkinson's disease (Espin et al.1997).

ACT is a second generation of synthet).ic mono-aromatic retinoid, pharmacologically the active metabolite of etretinate (Ormerod et al. 2010). ACT has the potential to inhibit and control some processes such as cell differentiation and proliferation, inflammation and keratinization (Pilkington and Brogden 1992). Retinoids affect the development and differentiation of epidermal cells, as well as the activity of adipose tissue by acting on the nuclear receptors of these processes Like all retinoids, ACT has also teratogenic effect (Ling 1999). Previous researches showed that after treatment with ACT, most patients have many side effects and pathology of various organs occurs (Katz et al. 1999 ; Bhuiyan and Chowdhury 2016).

MTX is a drug whose chemical structure resembles that of folic acid and which is able to block cell metabolism (Seideman et al. 1993). Methotrexate has been used in the treatment of many autoimmune diseases because it has folate antagonistic, anti-inflammatory and anti-proliferative

properties (Gerards et al. 2003). It is also used in lung, breast, acute lymphoblastic leukemia, lymphoma, head and neck cancers because MTX inhibits cell division (Gerards et al. 2003). MTX can indirectly prevent the synthesis of purine bases, which are necessary in the synthesis of DNA, RNA and adenosine phosphate by inhibiting the activity of tetrahydrofolate enzyme. Thus, the regeneration of cells becomes difficult which resulting in cell death (Jeffes and Kaneshiro 1998). Therefore, MTX has been used to treat various types of malignancy (cancer). Today, they are increasingly used for treatment in dermatology and rheumatology especially in psoriasis, psoriatic arthritis and rheumatoid arthritis (Lagarce et al. 2015).

MTX and ACT are known to have a healing effect in the treatment of psoriatic lesions. However, the combination of ACT and MTX is very rare in the treatment of psoriasis because the interaction of drugs leads in particular to hepatotoxicity (Carretero et al. 2013). The treatment of psoriasis should be taken in drug combinations against these side effects. It is recommended to take antioxidants when combine use is necessary (Karadağ et al. 2015).

ALA, which is found naturally in human foods, has antioxidant and anti-inflammatory activities (Rochette et al. 2013). ALA may have functions such as regeneration of vitamins C and E, protection against lipid peroxidation and free radicals, increasing SOD and CAT enzyme activities, repairing molecular damage, increasing acetylcholine production (Suh et al. 2005) In recent years, although the side effects are known, the use of the combination ACT + MTX in treatment has increased (Carretero et al. 2013). In the light of this information, in this study, the effect of ACT+MTX combination on polyphenol oxidase enzyme activity in rat lung tissue and whether it has protective role of antioxidant ALA was investigated.

2 Materials and Method

In this experimental study, 50 Wistar albino type male rats (weighing between 250 and 300 g) were used. All the animals, which were maintained for 12 hours in light and 12 hours in the dark and at a temperature of 21° C, were obtained from Ondokuz Mayıs University, Experimental Animals Application and Research Center (DEHAM). The studies were approved by the Ethics Committee of Ondokuz Mayıs University (2018/ 13). The rats were fed with standard mouse food and water was given unlimitedly.

The organizations of experimental groups were as follows:

Group 1 (C group) : The control group is the group that has not been treated.

Group 2 (ALA group): received 50 mg/kg/day of ALA;

Group 3 (ACT + MTXgroup): received 20mg/kg/day of ACT and also received 20mg/kg/week of MTX;
Group 4 (ACT + MTX + ALA): received 20mg/kg/day of ACT, 20mg/kg/week of MTX and 50mg/kg/day of ALA.

There are 15 rats in each of Group 2, Group 3, Group 4 groups that make up the experimental groups. Rats were starved for the last 24 hours before injection. Injection procedures were carried out at the same time every morning. ACT, MTX and ALA were resolved in 0.9 % NaCl, ACT (20 mg /kg /day) (An et al. 2017), MTX (20 mg /kg /week) (Jingang et al. 2017), ALA (50 mg / kg / day) (Maritim et al. 2003) and their combinations were given to the rats as intraperitonal injection (i.p) at the body weight level of each. From each the of experimental groups, 5 rats on the 3rd day, 5 on the 5th day and 5 on the 7th day following injection were sacrificed by cervical dislocation by giving general anesthesia.

Rats were given 50 mg/kg ketamine HCl (ketalar) and 10 mg/kg Xylazine (Rompul) as general anesthesia. Following this, perfusion was performed with 0.9 % NaCl and lungs were removed. The lungs were placed in containers containing isotonic sucrose and stored in a freezer at -80 ° C for experimental procedures. After the lungs removed from the freezer were thawed, their weights were determined by weighing them on a sensitive scale. Then, homogenization, sonication and centrifugation processes were carried out according to the procedures. The supernatants obtained after centrifugation were used for analysis.

The activity of polyphenol oxidase in lung homogenates was determined by the Hung and Boucias (1996) method. 50 µL homogenate was rapidly added to 950 µL phosphate buffer solution containing 20 mM L-DOPA. Then, the activity was determined by reading the absorbance change in a minute against the curve at 420 nm. 1 Enzyme Unit: It was defined as a 0.001 increase in 1 minute in the tub where the reaction occurred.

All data were evaluated using SPSS 22.0 statistical software. The difference between groups was checked with Kruskal Wallis test.

3 Results

Compared to the control group, it was determined that phenol oxidase activity increased in the group given ALA. This increase in activity; While it was 24% ($p > 0,05$) on the 3rd day, it doubled on the 5th day. On the 7th day, it was observed that the 5th day activity increased approximately 10% ($p > 0,05$) (Fig 1).

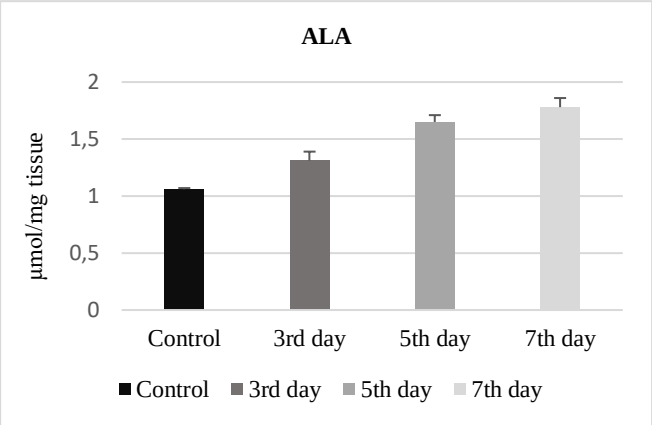

Fig. 1 Changes in Phenol Oxidase activity in the group receiving ALA

When MTX + ACT group is compared with the control group; While phenol oxidase activity increased 66% ($p <$

0,05) on the 3rd day, it was observed that it reached almost 100% ($p < 0,05$) on the 5th and 7th days (Fig 2).

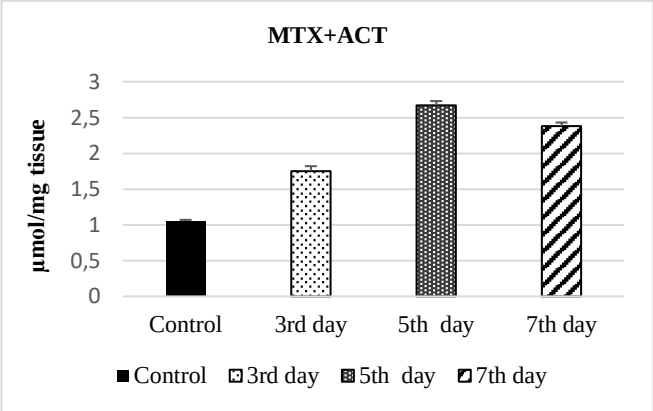

Fig. 2 Changes in Phenol Oxidase activity in the group receiving MTX+ACT

When MTX + ACT group is compared with the control group; Phenol oxidase activity increased 36 % ($p < 0,05$) on the 3rd day, % 12 ($p>0,05$) on the 5th % 82 ($p < 0,05$) on the 7th days (Fig 3).

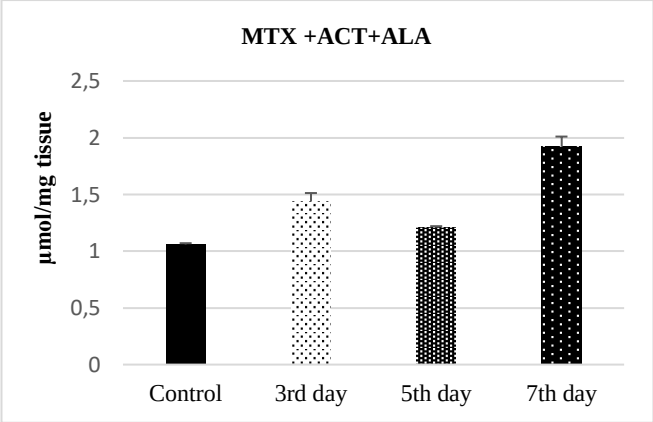

Fig. 3 Changes in Phenol Oxidase activity in the group receiving MTX+ACT+ALA

Phenol oxidase, which shows max activity on the 5th day in the MTX + ACT group; It was observed that reduced by 55% ($p < 0,05$) with the addition of ALA group (Fig 4).

4 Discussion

This study investigated the effect of acitretin and methotrexate application on phenol oxidase enzyme activity in rat lung tissues and as a result of adding alpha lipoic acid to this combination. Preview study has shown that oxidative stress caused by MTX plays a major role in tissue damage and these damages are reduced by ALA (Arpag et al. 2018).

MTX and ACT are widely used in the treatment of psoriasis. However, some case reports have shown that many side effects, especially acute interstitial pneumonitis, pulmonary fibrosis, occur after treatment with MTX (Bartram, 1998; Chikura et al. 2008).

In a study conducted by Armagan et al. in 2015, an increase in MDA level occurred in the group where MTX was given in kidney tissues compared to control. In the treatment group that received MTX + ALA, this increase decreased. It was concluded that ALA is effective with antioxidant and other properties in preventing the toxic effect of MTX in kidney tissue.

In the study of Moungjaroen et al. (2006), it has shown that ALA can produce reactive oxygen species that support ALA-related cell death in lung cancer. Gerritsen et al. (1994) explain that tyrosinase shows genoprotective activity by hydroxylation of tyrosine to L-DOPA and oxidation of L-DOPA to quinone. Han et al. (1996) stated that L-DOPA regulates the cellular antioxidant defense mechanism under certain conditions. Tyrosinase, peroxidase and laccase, members of the polyphenole oxidase enzyme family, have been investigated to remove toxic chemicals from drinking water and industrial wastewater (Wada et al 1992, Wada et al. 1993). However, many studies have been conducted, but activity studies in human metabolism have been limited. In our study, it was observed that MTX + ACT combination increased phenol oxidase activity in rat lung and with the addition of ALA to this combination, phenol oxidase activity decreased due to ALA. As a result, in this study, it was seen that ALA has a protective effect against the damage caused by MTX + ACT on rat lung tissue.

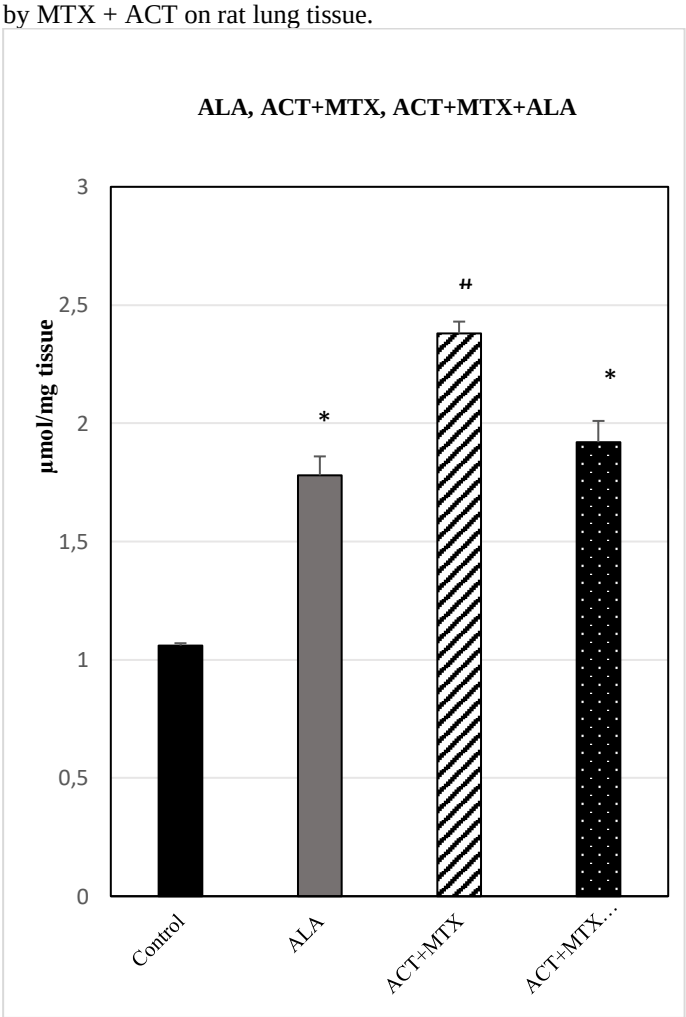

Fig. 4 Changes in Phenol Oxidase activity in the groups receiving ALA, MTX+ALA and MTX+ACT+ALA

(*$p<0.05$, ALA group compared to the control group), (#$p<0.05$, ACT+MTX group compared to the control group), (**$p<0.05$, ACT+MTX+ALA group compared to the control group)

5 Conclusion

In the study, it was observed that the combined use of ACT and MTX increased phenol oxidase activity in rat lung tissue. A decrease in this activity was observed with the addition of an antioxidant ALA to this combined application. When these results are evaluated, it can be said that MTX + ACT causes phenolic compounds in the lung tissue and ALA has a protective effect against MTX+ ACT derived phenolic compounds.

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Investigation of cloning strategies for the recombinant expression of a putative immune modulator TIR domain protein from probiotic *Lactobacillus casei* 21/1

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Abstract: TIR domain proteins have a key role in Toll-Like Receptor (TLR) signalling pathway in innate immunity. Bacteria can produce TIR domain proteins and those from pathogens were shown to manipulate TLR signaling via mimicking host proteins. Probiotics can also affect TLR signaling, but the molecular details have not been yet elucidated. In this study, a putative protein from probiotic *Lactobacillus casei* was identified as a TIR domain protein (LcTIR) based on sequence conservation. Multiple sequence alignments showed that LcTIR has high similarity to known TIR domains and the structural model of LcTIR verified the presence of the TIR domain fold. Following this, the gene encoding LcTIR was cloned in several *Escherichia coli* plasmids in order to obtain pure protein for structural and biochemical studies. Several fusion partners, promoter systems, different *E.coli* host strains and induction conditions were investigated to achieve recombinant protein production. In all conditions, recombinant LcTIR was produced at low amounts. The highest amount of protein obtained was GST-LcTIR fusion; in Rosetta(DE3)pLysS cells at 37°C with 0.5mM IPTG induction, where nearly all the protein was found in inclusion bodies. Furthermore, for all the constructs and strains tested, the low amount of LcTIR production suppressed cell growth and this might indicate its potential as an antimicrobial agent which opens a new era on bacterial TIR domains. This study is one of the first studies investigating the presence of probiotic TIR domain proteins, and future studies are needed to obtain soluble protein to assay their effect on TLR signalling mechanisms.

Keywords:TIR domain proteins; molecular cloning; probiotic; structural bioinformatics; toxic recombinant protein

1 Introduction

In recent years, studies on intestinal microbiome have increased. Interaction mechanisms of both pathogens and probiotics with intestine attract many researchers. Innate immunity plays a pivotal role when the system encounters microorganisms. Pattern recognition receptors (PRR) recognize microbial molecular patterns (MAMP) and initiate innate immune responses. Toll-like receptors (TLR) are a group of PRRs and function in recognition of several microorganisms and controlling the first immune response. Organization of TIR (Toll/interleukin-1 receptor) domain proteins in this pathway are very important in the transmission of the signal (O'Neill and Bowie 2007).

The TIR domain has a conserved fold, consisting of five parallel beta sheets in the center surrounded by alpha helices and loop regions. TIR domain is found in the structure of both TLR receptors (TLR1-10) and adaptor proteins (MyD88, TRIF, TIRAP (MAL), SARM and TRAM) in the cytoplasm. TIR domain proteins show high amino acid sequence similarity at especially conserved regions, and TIR domain proteins can be identified by sequence comparison (Ve et al. 2015). Such bioinformatics analyses showed that all kingdoms of life possess TIR domain proteins; including bacteria (Turner 2003). Studies were mostly focused on TIR domains from pathogenic organisms. It was shown that TIR domain proteins from several pathogens were able to interact with human TIR domain proteins (MyD88 and TIRAP) and this resulted in suppression of TLR signalling (Chaudhary et al. 2012; Cirl et al. 2008; Kaplan-Türköz 2017; Newman et al. 2006; Radhakrishnan et al. 2009; Salcedo et al. 2008; Snyder et al. 2013). These proteins have the conserved TIR domain fold (Kaplan-Türköz et al. 2013) and with this structural mimicry, they can interact with human TIR domain proteins, resulting in manipulation of the signaling pathway.

Recent research points out evidence that probiotics can also manipulate TLR signaling, but the mechanism has not been completely elucidated. Studies have shown that some probiotic strains can regulate mostly TLR2, TLR4, TLR9 and TLR5 signaling pathways. *Bifidobacterium breve* C50, *B.breve* Yakult and their cell free components were shown to induce production of IL-10 (anti-inflammatory cytokine) through TLR2 (Hoarau et al. 2006; Jeon et al. 2012). *Lactobacllus casei* CRL 431 were shown to induce IL-6 production through TLR2 signaling (Galdeano et al. 2007). *L.casei* Zhang was shown to increase the expression of TLR2 and TLR9 and reduce production of proinflammatory cytokines in a rat model (Wang et al. 2016). Similarly, an

increased expression of TLR2, TLR4, TLR5 and TLR9 in intestinal epithelial cells were detected in the presence of *L.casei* (Castillo et al. 2011).

Probiotic DNA was shown to be responsible for the modulation of TLR9 signaling pathways (Kitazawa et al. 2003). However, the molecular mechanism of probiotic action on other TLR pathways has not been illuminated. According to our genome analysis, we found gene regions encoding TIR domains in probiotic genomes and our hypothesis is that probiotics can also produce TIR domain proteins which can interact with human partners to regulate TLR signaling pathways.

2 Materials and Method

2.1 Materials

E. coli strains TOP10, BL21(DE3), BL21Star(DE3), BL21(DE3)pLysS, Rosetta2(DE3), Rosetta(DE3)pLysS cells were used in the study. LB broth (10 g/L Tryptone, 5 g/L Yeast Extract, 10 g/L NaCl) was used for cell growth. Antibiotics were added when appropriate; ampicillin (100 μg/ml), kanamycin (50 μg/ml), chloramphenicol (34 μg/ml). All chemicals were obtained from Merck or Sigma. All molecular biology reagents and kits were obtained from Thermo Scientific.

2.2 Bioinformatics methods

BLAST was used to identify probiotic origin TIR domain proteins (Madden 2002). Brucella TIR domain protein BtpA (pdb id: 4lzp) was used as query sequence for BLAST (blastp suite) and the search was restricted with *Lactobacillales* (taxid: 186826) taxonomic group to reveal probiotic origin TIR proteins.

Multiple sequence alignments between bacterial and human TIR domains were done using T-COFFEE (Expresso) (Notredame et al. 2000). The structural homology model of *Lactobacillus casei* 21/1 TIR domain (LcTIR) was built by RaptorX using *Paracoccus denitrificans* TIR protein (PdTIR) (PDB id: 3h16A) as the template protein (Kallberg et al. 2016). Structural models were displayed using graphics software PyMOL.

2.3 Cloning and protein expression

The genomic DNA of *L.casei* 21/1 was a gift from Prof. Dr. James L. Steel (Wisconsin University). Putative TIR domain gene region from *L.casei* 21/1 (507 bp) was obtained by PCR and the PCR fragment was cloned into several different plasmids (Table 1) and *E. coli* TOP10 cells were transformed with the recombinant plasmids.

Table 1 Properties of used plasmids

Plasmid	Antibiotic resistance	Promoter	Copy number
pET151/D-TOPO	Ampicillin	T7	Low
pQE80L	Ampicillin	T5	High
pETM11-SUMO3GFP	Kanamycin	T7	Low
pGEX-4T-2	Ampicillin	tac	Low

In order to express LcTIR, three different tag and/or fusion partners were tested; 6-Histidine tag (6His), small ubiquitin-related modifier (SUMO) and glutathione S-transferase (GST) fusion proteins. Some properties of the expected proteins are summarized in Table 2.

Table 2 Properties of expressed proteins

Plasmid	Expressed protein	Length (amino acid)	MW (kDa)	pI
pET151/D-TOPO	6His-LcTIR	201	23	5.05
pQE80L	6His-LcTIR-2	180	20	5.30
pETM11-SUMO3GFP	6His-SUMO-LcTIR	276	32	5.15
pGEX-4T-2	GST-LcTIR	399	46	5.24

Single colonies carrying the recombinant plasmids were grown in 5 ml culture medium overnight at 37°C and 150 rpm shaking. The next day 2% of culture was inoculated to fresh medium and were incubated at 37°C until an OD_{600} of 0.7- 0.9 and induction was done by adding 0.5-1 mM IPTG. The cultures were grown at 37°C for 6 hours or 25°C or 20°C overnight after induction and were monitored for protein expression using SDS-PAGE and Western Blot. After induction, cells were pelleted and equal number of cells were resuspended in loading dye, boiled and applied to 12% polyacrylamide gels. Gels were stained with either Coomassie brilliant blue R250 and/or blotted to PVDF membranes for 6His tagged proteins. Monoclonal anti-polyHistidine antibody (mouse) and anti-mouse IgG-alkaline phosphatase antibody and BCIP/NBT substrate were used for detection.

2.4 Protein solubility test and small scale protein purification

Solubility test and affinity purification were done for GST-LcTIR protein. Briefly, culture was centrifuged at 6500 rpm for 10 minutes at 4°C and the cell pellet was stored at -86°C. Pellets were resuspended in lysis buffer (50 mM Tris pH 8.0, 300 mM NaCl, 10 mM $MgCl_2$. 10% Glycerol, 1 mg/ml Lysozyme, 10 μg/ml DNase, 1 mM DTT, 1X Protease inhibitor) and incubated for 20 minutes on ice. Equal volume glass beads were added and vortexed for lysis. 1% Triton X-100 was added on the lysate and was centrifuged at high speed (16000 g for 20 minutes at 4°C) to separate soluble and insoluble fractions. The insoluble pellet was resuspended with lysis buffer (insoluble fraction) and the cleared supernatant (soluble fraction) was diluted with dilution buffer (50mM Tris pH 8.0, 5% Glycerol). For affinity purification, soluble fraction were mixed with glutathione resin which was previously equilibrated with Buffer A (50mM Tris pH 8.0, 150mM NaCl, 5% Glycerol) and allowed binding for 1 hour on ice with gentle rocking. After incubation the suspension was loaded on a disposable

column and flow through was collected. The column was washed with Buffer A and 10mM reduced glutathione pH 8.0 in Buffer A was used for elution.

3 Results and Discussion

3.1 Bioinformatics analysis and structural comparison

Our bioinformatics analyses have shown the presence of a hypothetical protein (LCA211_1668) in the genome of *Lactobacillus casei* 21/1 and this protein was named as LcTIR. The protein sequence was aligned with those of several different TIR domain proteins (Fig 1) and was found very similar especially in the conserved regions. Considering the sequence similarities given in Table 3, the high similarity of LcTIR sequence to bacterial TIR sequences is remarkable. In order to compare the similarities at the structural level, homology model of LcTIR was built using Raptor X (Fig 2a). The model contains five parallel beta sheets in the core and they are surrounded by alpha helices and loop regions, which is the conserved TIR domain fold (Ve et al. 2015). The LcTIR model is considered of high quality according to the criteria of the modeling software with P-value of 1.22e-04 and uGDT of 105.

Conserved regions (CR) for TIR domains were shown on LcTIR model (Fig 2b). Buried beta sheets on CR1 which are responsible of structural stability in mammalian TIR proteins (Ve et al. 2015), are similarly located in buried center at LcTIR model. Two loop regions; BB-loop and DD-loop, were shown to significantly contribute to TIR:TIR interactions which is necessary for TLR signaling (Zhang et al. 2012) These loops are located on the surface of LcTIR model and therefore has potential to contribute to protein interactions (Fig 2b).

LcTIR model was aligned structurally to other human and bacterial TIR domain proteins (Table 3) and the fit to bacterial origin TIR domains highlight the high degree of structural similarity (Fig 2c).

Table 3 Structural and sequence similarity between LcTIR and some other TIR domains

	Structral alignment with LcTIR model RMSD (A°)	Sequence identity with LcTIR sequence (%)
Bacterial TIR domains		
PdTIR (3h16)	0.580	35.56
BtpA (4lzp)	0.394	40.14
Human TIR domains		
MyD88 (2z5v)	1.405	26.67
TIRAP (2y92)	3.140	19.23
TLR1 (1fyv)	3.981	35.56
TLR2 (1fyw)	3.659	23.08
TLR6 (4om7)	2.859	25.23
TLR10 (2j67)	2.872	25.22

Protein production optimization

The gene encoding LcTIR was cloned into several different expression plasmids (Table 2) in order to produce enough protein for purification and characterization studies. The gene contains six rare codons, thus 6His-LcTIR protein expression studies were initially done using Rosetta2 (DE3) cells (Fig 3a and 3b).

6His-LcTIR protein was induced from pET151D-TOPO at three different temperatures (20°C, 25°C and 37°C) with 1mM IPTG and western blot showed that the amount of produced protein was very low, with no protein at 20°C (Fig 3a). Interestingly, the amount of protein did not increase with time and the biomass did not increase after 1.5 hours of induction (Fig 3b). These results suggested that LcTIR might be toxic to *E.coli* cells. In order to solve the toxicity problem, BL21(DE3)pLysS cells were used for expression and the amount of protein produced at 37°C were higher (Fig 3c). In order to increase the amount of protein, different IPTG concentrations were tested to control the toxicity and protein amount relatively increased with 0.2 and 0.5mM IPTG (Fig 3d). Still, the protein produced was not enough for further studies.

Next, a plasmid with a weak promoter was tested in order to slow down toxic LcTIR production and therefore the cells could accumulate protein over time. 6His-LcTIR-2 protein was expressed from pQE80L at 37°C with 0.5mM IPTG induction using different expression cells. In all the conditions tested, protein amount did not increase over time and therefore, toxicity problem was not solved (Fig 3e).

Following these, different fusion partners were tested in order to decrease the toxicity of LcTIR. 6His-SUMO-LcTIR was expressed from pETM11SUMO3GFP vector at 20°C with 1mM IPTG induction (Marblestone et al. 2006) (Fig 3f), or at 37°C with 0.5mM IPTG induction (Fig 3g). Relatively moderate amount of protein was obtained from Rosetta (DE3) pLysS cells, however, the problem of toxicity still persisted and protein sufficient for purification was not obtained.

Another recommended fusion partner for solving the toxicity problem is GST (Saluta 1998). *E.coli* cells carrying pGEX4T2-LcTIR plasmid were not affected by protein expression, as cell growth continued to increase at different temperature and inducer concentrations (data not shown). Furthermore, the amount of protein was shown to increase after induction. Therefore GST fusion seemed to eliminate the toxic effect of LcTIR. Although the molecular weight of the expected protein is 46kDa, the observed protein in the polyacrylamide gel appeared just below 40kDa (Fig 3h).

Although the reasons for this cannot be fully explained in the literature, it has been reported that GST fusion protein might denaturate under reducing conditions in SDS-PAGE and it can position below expected in the gel (ThermoFisher 2019). The highest amount of protein was obtained at 37°C, with 0.5mM IPTG, from Rosetta (DE3) pLysS cells (Fig 3h).

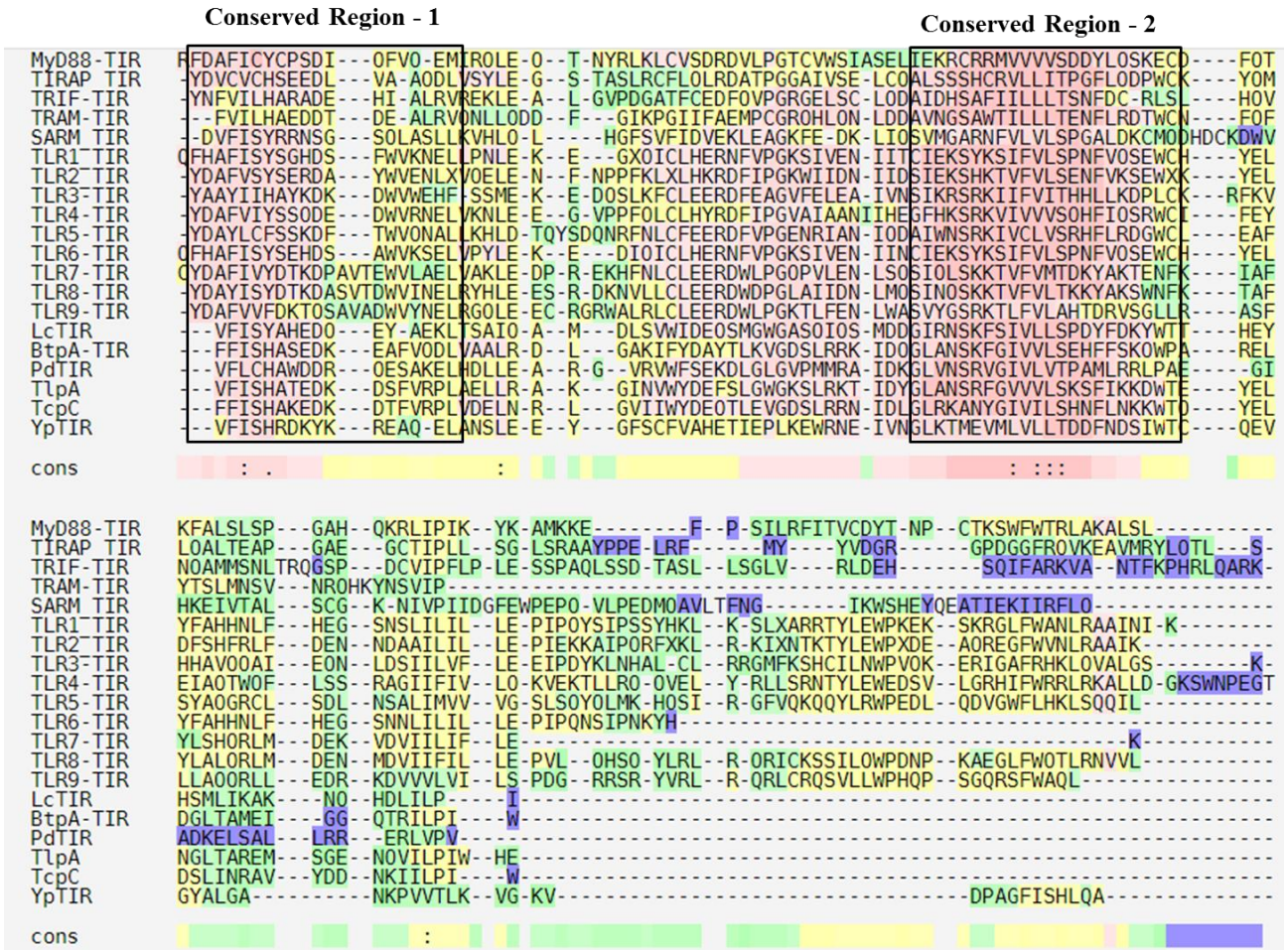

Fig. 1 Multiple alignment of human TIR domains, probiotic TIR domain LcTIR and bacterial TIR domains using T-COFFEE. NCBI or PDB id of the sequences used in the alignment: human origin; MyD88 (2z5v), TIRAP (2y92), TRIF (2m1x), TRAM (2m1w), SIGIRR (CAG33619.1), TLR1 (1fyv), TLR2 (1fyw), TLR3 (ABC86908.1), TLR4 (AAF89753.1), TLR5 (AAI09119.1), TLR6 (4om7), TLR7 (AAZ99026.1), TLR8 (AAZ95441.1), TLR9 (ACQ41824.2), probiotic origin; LcTIR (EKQ02965.1), pathogenic bacterial origin; BtpA (4lzp), PdTIR (3h16), TlpA (WP_000028416), TcpC (ADO30448), YpTIR (KNX91904.1). Conserved regions are shown with boxes.

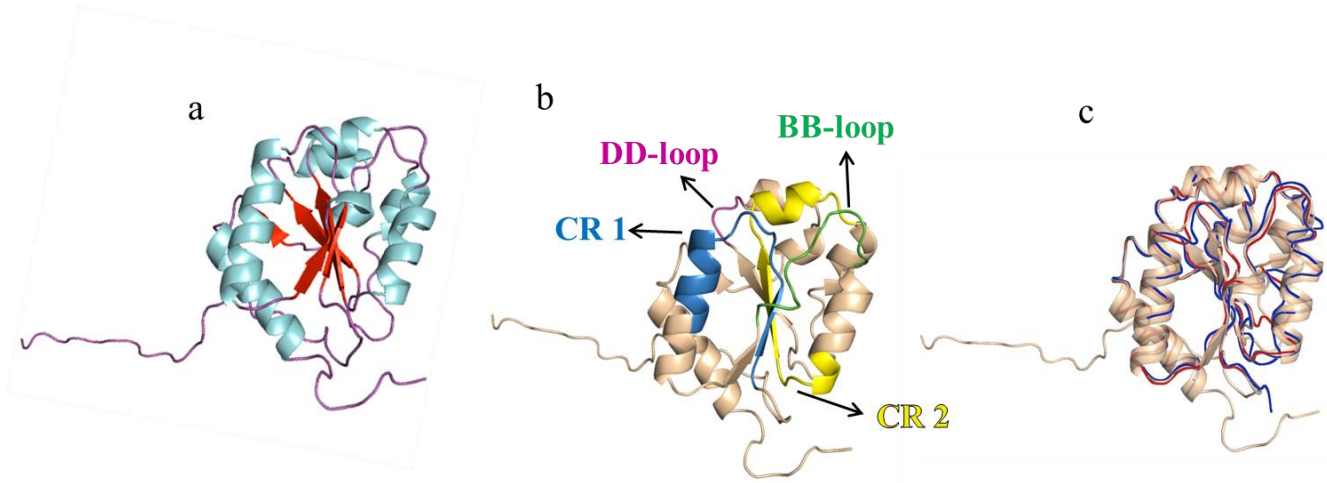

Fig. 2. LcTIR homology model. a. Ribbon display, colored for secondary structures; beta sheets:red, helices:blue, loop regions:purple. b. Conserved regions (CR) displayed on LcTIR model; CR 1 : blue, CR 2: yellow. BB-loop: green, DD-loop:pink. c. Structural alignment LcTIR and bacterial TIR domains; LcTIR shown as cartoon (wheat), PdTIR (3h16) blue ribbon and BtpA (4lzp) red ribbon.

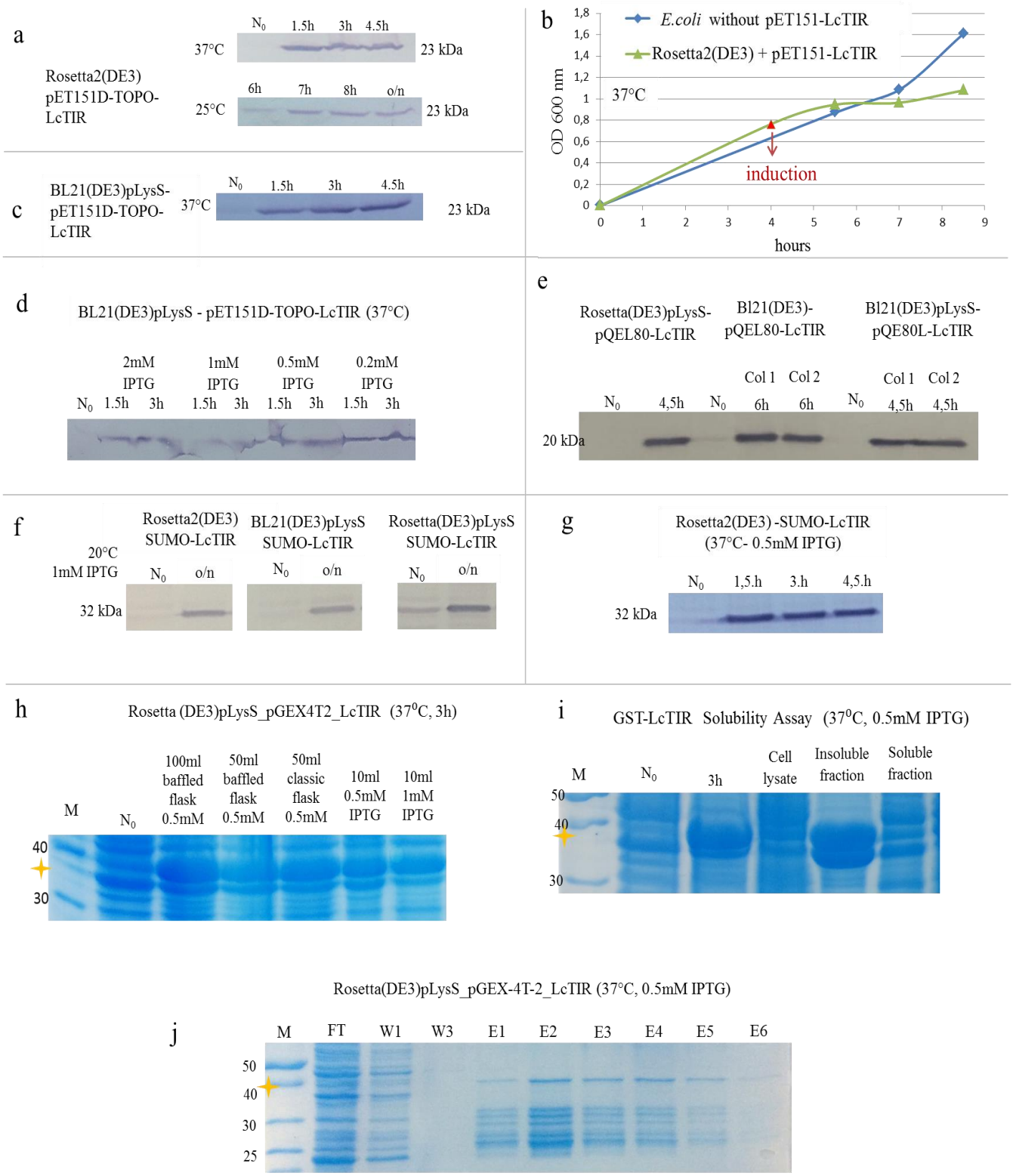

Fig. 3. Recombinant LcTIR production from different plasmids and expression hosts. a,c,d,e,f,g: Western blot detection of different His-tagged LcTIR fusion. b: Growth curve of cells expressing 6his-LcTIR (green) and cells without recombinant LcTIR plasmid (blue). h,i: Coomassie blue staining of GST-LcTIR SDS-PAGE GST-LcTIR bands are shown with a yellow star.. M: marker,N_0: before induction , o/n:overnight, Col: colony, baffled: baffled flask, classic:classical flask. j: Affinity purification fractions of GST-LcTIR analyzed on SDS-PAGE. FT: flowthrough, W:wash, E:elution fractions

Large scale protein production was done for the purpose of purification. However, most of the protein was found in the insoluble fraction; inclusion bodies; as shown by solubility test (Fig 3i). Thus, GST-LcTIR was not toxic to the cells as the protein was trapped in the inclusion bodies. Purification was performed with the small amount of soluble protein. Elution fractions contained very low amount of GST-LcTIR, not enough for further studies (Fig 3j).

For all the above mentioned recombinant plasmid-expression cell combinations, several colonies were screened but no difference was observed among them. In addition, other suggestions in order to overcome the toxicity problem; plating method (Suter-Crazzolara and Unsicker 1995), expression in terrific broth or LB broth containing 3% ethanol (Chhetri et al. 2015) were tested, with no success (data not shown).

Toxic protein is a common challenge in recombinant expression systems and various ways to solve this have been proposed (Ahmad et al. 2018; EMBL 2019; Kaur et al. 2018). In this study several methods such as, using pLysS cells, expression from weak promoter and fusing with partners were tested. There are other suggested methods such as; using pBAD promoter which with protein production is more tightly controlled (Rosano and Ceccarelli 2014), expression with other fusion partners like NusA, Trx, MBP, Fh8 (Costa et al. 2013; Rosano and Ceccarelli 2014), periplasmic expression (Bloois 2012) and using C41 (DE3) or C43 (DE3) cells for toxic protein production (Wagner et al. 2008). Also different expression systems; lactic acid bacteria, yeast or insect; can be evaluated (Song et al. 2017; Tripathi and Shrivastava 2019). Another suggestion is purification from inclusion bodies and refolding (Rosano and Ceccarelli 2014). However, it was not preferred for LcTIR protein since there is no verified method to test functional properties after refolding.

4 Conclusion

In this study, a putative TIR domain protein from a probiotic strain of *L.casei* was identified using bioinformatics methods. Sequence and structural alignments of the protein showed its similarity to other TIR domain proteins Therefore, LcTIR can be considered to have potential to mimick human TIR domains and regulate TLR signaling similar to other bacterial TIR domain proteins.

In order to investigate this potential, attempts were made to produce recombinant LcTIR from *E. coli* cells. After several different conditions, it was shown that protein has a toxic effect on cells and therefore could not be produced at high quantities. The highest amount of protein obtained was GST-LcTIR fusion; from Rosetta(DE3)pLysS cells, where nearly all the protein was found in inclusion bodies.

This study is one of the first studies on probiotic origin TIR domains, and future work will contribute to our understanding of probiotic effect on TLR signaling.

Studies are underway to investigate other cloning methods including expression as MBP fusion and recombinant expression from *Lactococcus lactis* to produce and purify LcTIR for further characterization studies. These characterization studies will focus on solving the solution structure using small angle X-ray scattering and crystal structure via X-ray crystallography. Furthermore, it will be necessary to investigate the *in vitro* interaction of LcTIR with human TIR domains using pull down assays and gel filtration chromatography.

These studies will show the functionality of the probiotic TIR domain and will pave the way for the production of targeted probiotic molecules for food and medicine applications.

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Extraction of collagen and gelatine from animal wastes

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Abstract: Collagen is the structural protein found in connective tissues in mammals and comprises about 60% of whole body protein. Gelatine is the protein product obtained from collagen via various procedures such as thermal denaturation, partial hydrolysis and chemical treatments. Animals such as bovine, sheep, chicken and fish are most commonly used for the production of foods for human nutrition. The wastes such as skins and bones raised during manufacturing of meat products are considered as rich sources for collagen. Various procedures are developed to extract collagen and gelatine as well from those wastes. The resultant protein materials with different purities can be served as value added products promising various application fields based on their characteristics. Many types of collagen are present based on structural differences however, most of the collagen in the body consists the types of I, II and III. Type A and type B are also the two common types of gelatine derived from collagen via acid or alkali treatments at different isoelectric points. Differences in amino acid composition can be encountered in different gelatine types obtained from different sources as well. This study briefly summarizes recovery of collagen and gelatine from animal wastes. Well-defined collagen and gelatine obtained from those wastes can be used to develop biomaterials available for cell scaffolding in tissue engineering besides serving opportunity in manufacturing edible products.

Keywords: collagen, gelatine, purification, animal wastes

1 Introduction

Gelatine produced by partial hydrolysis of collagen is not considered as a food additive in our country as in many countries (Anon, 2008). Gelatine is used for the development of textural and rheological properties in various foods (Baziwane and He, 2003). Due to its many technological features, gelatine is widely used in the production of food cosmetics, medicine, photography, pharmacy and agricultural products (Yetim, 2011). Collagen, the raw material of gelatine, is a structural protein that is quite common in animals. It is found in high amounts in tendons, skin and bones (Balian and Bowes, 1977; DeMan, 1999). These wastes that occur during the processing of animals such as cattle, sheep, goats and poultry after slaughter can be shown as an inexpensive and potential source for gelatine production. The gelatine obtained from those has a wide range of uses due to its technological and functional properties. This review aims to give brief information about recovery of collogen and gelatine from various animal wastes.

2 Raw material of gelatine: collagen

Collagen is one of the most common proteins in animals. Its ratio to all proteins in the body is %60 (Aberle et al. 2001; Schrieber and Gareis 2007). Collagen fibers consist of protein called tropocollagen. With the crystallization of tropocollagen, microfibrils, collagen fibrils from microfibrils, and collagen fibers from collagen fibrils. There are 27 different types of collagen. Type 1 collagen is found is bones, skin and tendons, type 2 colagen cartilage tissue and type 3 collagen in young tissues (Gomez-Guillen et al., 2011). The structure of collagen contains %12 proline, %11 alanine, %35 glycine and %9 hydroxyproline. The most important feature that distinguishes collagen from other proteins is the proline and hydroxyproline ratio (Gözükara 2001). The amount of hydroxyproline directly gives information about the amount of collagen and/or the amount of gelatine. There are cross-molecular cross-links in collagen. The number of these bonds increases as the animal ages. With the collagen molecule aging, trivalent bonds are established instead of divalent. Soluble gelatine can be obtained by subjecting these crosslinks to a more severe process (Ledward 2000). Collagen, which is the raw material of gelatine, is a water-insoluble protein with a molecular weight of 330 kDa, unlike gelatine.

3 Gelatine

Gelatine is a protein produced by controlled hydrolysis of collagen from the skin, bone and connective tissues of animals such as cattle, sheep, goats, pigs, poultry and fish. Gelatine, with 60-65 kDa in weight, is produced by breakig the cross bones in the collagen molecule weighing 300-500 kDa (Ockerman and Hansen 1988). It consists %51 carbon, %7 hdyrogen, %25 oxygen, %17 nitrogen molecules. About %85-92 of the gelatine molecule is consisted of the collagen molecule (Nur Hanani et al. 2014). Gelatine considered as GRAS (generally regarded as safe) status has superior properties compared to other gelling carbohydrates. It has properties such as thickener, gelling, emulsifier, foaming and film forming. It is easy to digest and can melt at the body temperature of human. Besides, gelatine can also provide numerous health benefits such as maintaining body tissues, giving healthy and youthful appearance to skin, strengten joints and bones, improving hair and nail growth, and helping to regulate blood sugar (Fernandez and Perez 1998; Rubio et al., 2008). Since gelatine has many technological, functional and health promoting features, it has a wide range of applications in food industry and medicine as well. The fact that enabling to produce from cheap and available raw materials affects the availability of gelatine positively.

4 Gelatine production

Gelatin production for targeted purposes includes sequential steps of raw material preparation, demineralization, acid and alkali treatments, and solvent extraction with varying process conditions. Major stages are given below and figure1 presents a general scheme for obtaining gelatine from animal wastes.

4.1 Raw (waste) materials

Cattle, sheep, goats, poultry and pig skin and bones are mostly used as raw materials for the production of collagen and gelatine. Hairs, blood, meat residues, fat and connective tissues were removed from bones and skins before the treatment. They are cut into small pieces in order to increase surface area and enhance recovery. In case of bone samples, calcium carbonate in the structure is removed with the help of concentrated HCl solution. As a result of demineralization, bone material called as ossein is obtained. After the raw materials are prepared properly, they are ready for acid and alkali treatments in the following step.

4.2 Alkali and acid application (pre-treatments)

Cross links in the structure of collagen are broken down by acid and alkali treatment. In the hydrolysis of collagen, the cross links are cleaved with dilute alkali and/or dilute acid, while the protein chains in the collagen remain intact (Hattrem et al. 2015). Type A and B gelatine produced from collagen are two common types of gelatine with different isoelectric points. The isoelectric point of A type gelatine is between 8,9 – 9,4 while B type gelatine is between 4,8 – 5,5. While chemical applications are made in the production of gelatine from collagen, a moderate temperature application can also be made. The cruciate ligaments which are broken down by chemical application and moderate temperature application have been prepared for extraction as well.

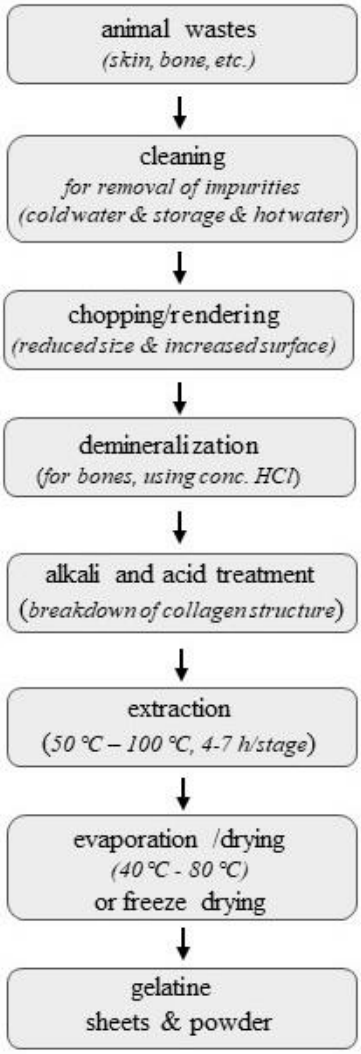

Figure1. A scheme of gelatine production from animal wastes

4.3 Extraction

Extraction is carried out with the help of acidic or alkaline solvents. Initial temperature can be accepted as 50 °C and it can be increased up to 100 °C in the following steps. Each stape takes an average of 4-7 hours. First, temperature applied is above the denaturation temperature of collagen (Erge and Zorba 2016). Oil emulsion is prevented by applying mixing during the extraction process. Due to the increase in temperature, maillard reaction may occur and dark colored gelatine could be obtained. (Duconseille et al. 2015). The extraction process is significantly affected by the age of the animal used, the particle size and the temperature applied. The age of animal and particle size prevent the occurrence of a homogeneous extraction process. By performing the extraction process at high temperature for a long time, high efficiency and gel strength can be obtained in the resultant gelatine product. In case of the lower temperature, extraction cannot occur properly and the yield decreases (Yetim 2011).

After extraction, gelatine containing solutions are filtered to remove oil and other suspended contaminants. Gelatine exract is concentrated by evaporation until it reaches a certain viscosity. High quality gelatine is aimed at about 20-25%, but if lower quality gelatine is targeted, it is reduced to around 40%. (Ledward 2000). Then gelatine is turned into gel by sterilization. Gelatine is pulverized by drying process. The moisture content of commercially produced gelatines varies between 8 and 12% (Yetim 2011). Physicochemical properties such as isoelectric points, moleculer weight distribution, gel strength, viscosity, melting and gelling temperature and color can be determined to assess the quality characteristics of the resultant gelatine product.

4.4 Physicochemical Properties of Gelatine

Physical and chemical properties of gelatine obtained after extraction should be evaluated in order to determine the quality of gelatine.

The amino acid composition of the gelatine is different from the collagen amino acid composition if treated with alkali. In alkaline treatment, non-ionized asparagine and glutamine amino acids turn into carboxyl groups. As a result, gelatine has an acidic property. For this reason, the isoelectric point of alkali-treated gelatine is between 4.8-5.5, and the isoelectric point of acid-treated gelatine is between 7.0-9.4 (Ledward 2000).

Gelatine produced after various processes consists of polypeptides with different molecular weights. The molecular weight distribution varies according to the non-hydrolyzed crosslinks in polypeptides in gelatine, intra-molecular and intermolecular covalent bonds in the material used as the source, and polypeptide chain length (Eysturskaro et al. 2010; Schrieber and Gareis 2007). High temperature and concentrated chemicals applied in the pretreatment and extraction stages cause the primary structure to break down and the molecular weight to decrease.

The molecular weight distribution of gelatine significantly affects viscosity, bloom value and melting/gelling temperature value. High molecular weight is the determinant of high viscosity, high bloom value and high gel strength (Ledward 2000).

Gel strength is an expression of gelatine gel resistance or hardness, and it is an important quality feature that allows gelatine to be classified as industrial. As bloom value of gelatine increases, market value and quality increase as well (Sarbon et al. 2013). Bloom value varies depending on the molecular weight distribution of gelatine. Molecular weight varies depending on the chemicals used and the temperature used in the pretreatment and extraction stages as well. In chemical processes under moderate conditions gelatine with high bloom value can be obtained. On the contrary, as a result of long-lasting and high-temperature processes gelatine with low bloom value can be obtained due to the hydrolysis of the polypeptide chains (Eysturskaro et al. 2010). Other factors affecting the bloom value of gelatine are the amount of proline and hydroxyproline amino acids, amino acid distribution and the source of collagen to be obtained.

Viscosity is one of the most important quality features of gelatine. Molecular weight distribution is one of the most important factors affecting the viscosity of gelatine. The viscosity increases as the molecular weight increases. The concentration and temperature of the chemicals applied in the pre-treatment and extraction stages affect the molecular weight of the gelatine, as well as the physicochemical properties such as viscosity (Uriarte-Montoya et al. 2011). The viscosities of the commercial gelatines can vary between 2-7 cP. Specially produced gelatines can have 13 cP viscosity. High viscosity gelatines are stronger but more flexible whereas low viscosity gelatines are weak and soft (Karayannakidis and Zotos 2014).

The temperature at which the gelatine gel becoming a solution is defined as the melting point, and the temperature at which the gelatine solution turning a gel is considered as the gelling point (Schrieber and Gareis 2007). Factors affecting the melting and gelling temperature of gelatine are bloom value, concentration, amino acid distribution, source of collagen and amount of proline and hydroxyproline.

The transparency and color of the gelatine obtained as a result of the processes is one of the quality criteria that significantly affects the technical properties, commercial capacity and consumer preferences (Du et al. 2013). As a result of the maillard reaction between the proteins and carbohydrates can provide medium the color of gelatine becomes brown. The intensity of this color can vary depending on the extraction time and temperature (Duconseille et al. 2015). Thus, the color of gelatine differs based on the formation of the maillard reaction, the increase in pH, the source of the gelatine and the concentration of chemicals used in the pre-treatment stage.

All of the physicochemical properties are important factors that determine the market value of gelatine. By calculating and explaining these factors quantitatively, gelatine is used in accordance with the intended purpose.

5 Conclusion

The need for gelatine is gradually increasing in the food industry. Wastes and by-products such as skin, bone, hair and tendon obtained from animal sources such as cattle, sheep, chicken and fish can be evaluated as the raw materials for collagen and gelatine production. However, as these raw material resources are not sufficient, searches for alternative resources are still ongoing. In addition to the food industry, gelatine can find wide use in the fields of medicine, cosmetics and pharmacy. Advantages that increase the use of gelatine are easy production, ability to melt at body temperature its own desirable brightness and transparency, GRAS status and being rich source of protein supporting human health. The disadvantages can be stated as the limitation of its use as an animal waste product in terms of religious rules. When the advantages and disadvantages are evaluated together, the researches on gelatine production and application are gradually increasing. Considering the physicochemical properties advanced researches are needed to optimize the yield and quality of gelatine.

Authors' contributions

This study is based on a part of Master Thesis of Ayşegül Gündem and Dr. Özgür Tarhan is the supervisor of her thesis. Equal contribution is provided by the authors.

Conflict of interest disclosure

The authors did not declear any conflict of interest.

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