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The Use of *Lactobacillus rhamnosus* Gg, *Saccharomyces Boulardii*, and *Pediococcus acidilacticii* C69 to Control Vancomycin-resistant Enterococci Colonization in a Rat Model

Sıçan Modelinde Vankomisine Dirençli Enterokok Kolonizasyonunu Kontrol Etmek için Lactobacillus Rhamnosus Gg, *Saccharomyces boulardii* ve *Pediococcus acidilacticii* C69'un Kullanımı

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Abstract

Objective: Vancomycin-resistant enterococci (VRE) are responsible for a considerable amount of healthcare-associated infections. In this study, we investigated the ability of three probiotic organisms to eliminate VRE colonization or protect against gastrointestinal (GI) epithelium-induced injury in a rat model.

Material and Methods: This was a prospective study and a rat model of gastrointestinal VRE colonization which was created in 28 rats, and 3 rats were separated without VRE colonization for the histological control group. Three probiotic organisms (*Lactobacillus rhamnosus* GG, *Saccharomyces boulardii*, and *Pediococcus acidilacticii* C69) were studied to investigate their ability on VRE colonization. On the 3rd, 5th, and 9th day (d), the densities of VRE and probiotic bacteria in fecal samples weremeasured. All animals weresacrificed on the 9th d of the study for histological examination.

Öz

Giriş: Vankomisin rezistan enterokoklar (VRE) sağlık hizmeti ile ilişkili önemli enfeksiyonlardan sorumludur. Bu çalışmada, bir sıçan modelinde üç probiyotik organizmanın VRE kolonizasyonunu ortadan kaldırma veya gastrointestinal (GI) epitelyumdaki hasara karşı koruma yeteneğini araştırdık.

Gereç ve Yöntemler: Bu prospektif bir çalışmadır, 28 sıçanda gastrointestinal VRE kolonizasyonu için bir sıçan modeli oluşturulmuş ve 3 sıçan histolojik kontrol grubu için VRE kolonizasyonu olmadan ayrılmıştır. Üç probiyotik organizmanın (*Lactobacillus rhamnosus* GG, *Saccharomyces boulardii* ve *Pediococcus acidilacticii* C69) VRE kolonizasyonu üzerindeki yeteneklerini araştırmak amaçlandı. 3, 5, ve 9. günlerde dışkı örneklerindeki VRE ve probiyotik bakterilerin yoğunlukları ölçüldü. Tüm hayvanlar histolojik inceleme için çalışmanın 9. gününde sakrifiye edildiler.

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Results: Gastrointestinal VRE load in the probiotic groups was significantly decreased more than the serum physiologic (SP) group on the 9th d (p= 0.021). The ratio of moderate to severe changes in the histological examination of tissue samples was significantly higher in the SP group as compared with the probiotic groups (p= 0.000). The histology of the *S. boulardii* group was better preserved than that in the other two probiotic groups.

Conclusion: None of the probiotic strains, despite reducing the load, could not eliminate VRE colonization. However, all probiotics protected against gastrointestinal epithelium damage and prevented VRE-associated injury. *Saccharomyces boulardii* was the most protective in gastrointestinal damage. The use of probiotics might be an alternative treatment way to prevent epithelial damage or colitis associated with VRE for researches.

Keywords: Enterobacteria, infection, intestinal microbiology, modeling, resistance

Bulgular: Probiyotik gruplardaki gastrointestinal VRE yükü, 9. günde serum fizyolojik (SP) grubundan önemli ölçüde azdı (p= 0.021). Doku örneklerinin histolojik incelemesinde orta-şiddetli değişikliklerin oranı SP grubunda probiyotik gruplara göre anlamlı derecede yüksekti (p= 0.000). *S. boulardii* grubunun histolojisi diğer iki probiyotik gruptan daha iyi korunmuştur.

Sonuç: Hiçbir probiyotik suş VRE kolonizasyonunu azaltsa da ortadan kaldıramadı. Bununla birlikte, tüm probiyotikler gastrointestinal epitel hasarına karşı koruma sağladı ve VRE ile ilişkili hasarlanmayı önledi. *Saccharomyces boulardii*, gastrointestinal hasar için en koruyucu olan suştu. Probiyotiklerin kullanımı VRE ile ilişkili epitel hasarını veya koliti önlemekte araştırmacılar için alternatif bir tedavi yolu olabilir.

Anahtar Kelimeler: Bağırsak mikrobiyolojisi, direnç, enfeksiyon, enterobakteri, modelleme

Introduction

Enterococci are found in the gastrointestinal tract and are responsible for a considerable amount of healthcare-associated infections, including urinary system tract infections, intraabdominal and pelvic reactions, skin and soft tissue infections, endocarditis, bacteremia, and neonatal sepsis (1,2). The common pathway for nosocomial vancomycin-resistant enterococci (VRE) transmission is via person-to-person contact or VRE-contaminated objects. Colonized patients are the most important reservoirs for VRE transmission. The majority of VRE colonization occurs in the gastrointestinal tract, but VRE can also be found to a lesser extent on other sites; including the skin, genitourinary tract, and oral cavity (2,3). Vancomycin-resistant enterococci can cause various infections in immunocompromised patients (3). Thus, preventing gastrointestinal tract colonization with VRE is an important strategy for limiting dissemination and infection. However, at present, there are no effective VRE decolonization methods, and recurrence of VRE may be observed days or weeks after the initial infection (1,2).

Probiotics are defined as living microorganisms (bacteria and yeast) resistant to digestion and reach the colon alive. Probiotics could also reduce proliferation rates of normal colonic mucosa of rats when the normal control mechanisms are still completely effective (4). They have been multiple types of health benefits. *Saccharomyces boulardii* (*S. boulardii*) is a non-pathogenic yeast and is one of the used biotherapeutic agents. Some clinical studies have shown the impact of *S. boulardii* on the prevention and treatment of various intestinal disorders. *Saccharomyces boulardii* is used in many countries of the world to prevent and treat diarrhea and other digestive diseases caused by the use of antibiotics. In the study carried out on diarrhea that related to infection; *S. boulardii*, *Lactobacilus acidophilus*, *Bifidobacterium bifidum* was highly effective (5). *S. boulardii* had been also found to reduce the duration and amount of diarrhea, which caused by an infection in children (6). *Lactobacillus rhamnosus* GG (*L. rhamnosus*) strain was effective especially in children with diarrhea due to rotavirus. In an animal experiment results had suggested that the synbiotics formulated with lactulose and *Pediococcus acidilacticii* (*P. acidilacticii*) have potential benefits to prevent and improve colibacillosis in piglets (7).

Current evidence supports the idea that probiotics have beneficial effects on the gastrointestinal tract. A wide spectrum of probiotics, including *L. rhamnosus* and *S. boulardii* is currently available in the U.S. and Europe (8). We hypothesized that probiotics would decrease the VRE colonization rate in a rat model. To shed light on this issue, we compared the microbiological and histological effects of three probiotic microorganisms (*Lactobacillus rhamnosus* GG (ATCC 53103), *S. boulardii* (Hansen CBS 5926), and Pediococcus acidilacticii C69) in a rat VRE colonization model.

Materials and Methods

Design

Our study was started after obtaining permission from a Medical School Hospital Experimental Animal Local Ethics Committee (Protocol Number: 114/2013). This prospective study consisted 31 female albino Wistar rats with average weights of 200-250 g. A rat model of gastrointestinal VRE colonization was created, as described in Section 2.2. Then, twenty-eight rats in which VRE colonization was confirmed by culture randomized into four groups. Extra 3 rats out of these 28 rats were separated without any serum physiologic and probiotic solution application to examine the normal histological structure in histological evaluation and comparison.

Rat Model

Three randomly selected female IOPS OF1A rats, none of which initially were colonized, were individually caged and screened for previous VRE colonization. We saw that they were VRE negative. Gastric gavage of 1.5 mg/ml of VRE (5 x 10^9 CFU (colony forming units)/ml) (with a total dose of 30 mg/20 ml/kg) standard solution was applied and colonization of VRE initially was observed in the first three rats (Figure 1). Later on, a total of 28 rats were colonized by VRE.

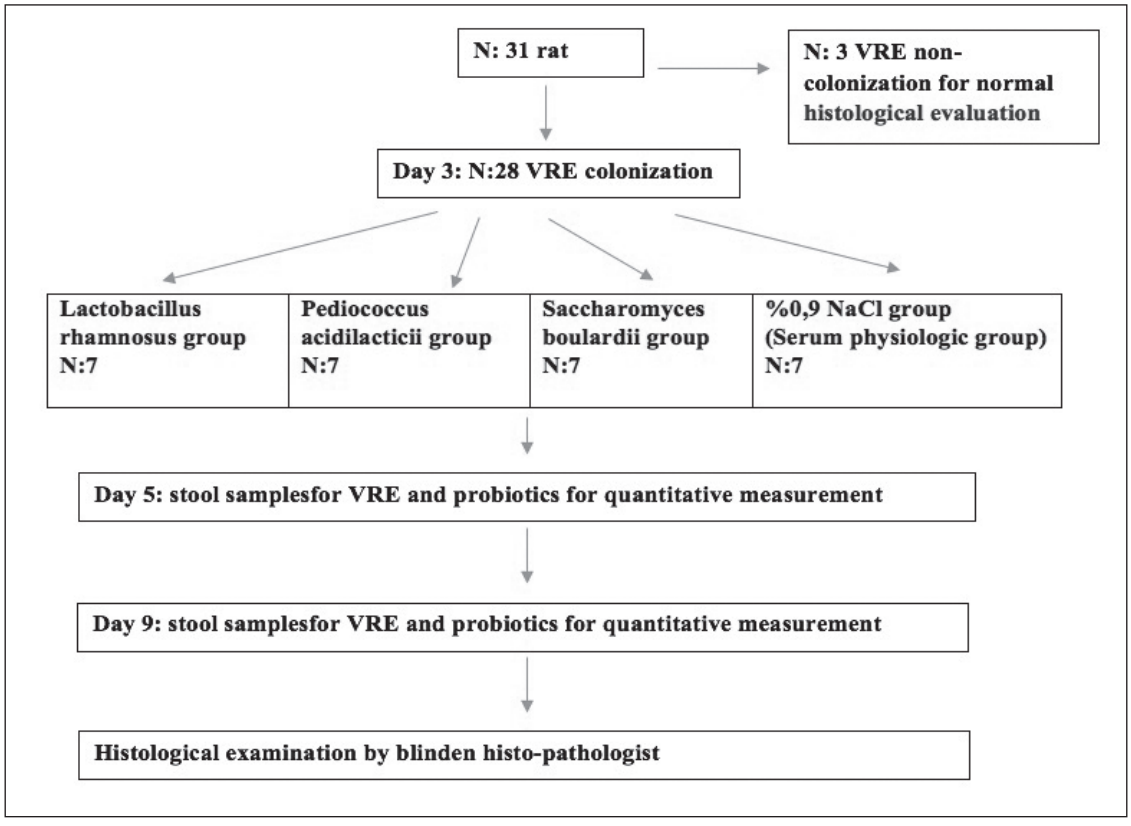

Figure 1. The general outline of the study plan.

Treatment Groups

We selected one of the subgroups of different species, probiotic strains that have been shown to affect the gastrointestinal tract by different mechanisms. We tested one probiotic from three different species under laboratory conditions and applied it as a standard solution and looked at the effects on VRE. We selected *L. rhamnosus* from Lactobacillus species, *S. boulardii* from Saccharomyces species, and *P. acidilacticii* from *Pediococcus* species; because they have been shown to have positive effects on the intestine and infectious diarrhea. The probiotic solution (*L. rhamnosus*, *P. acidilacticii*, and *S. boulardii*) (4 mg/ml in 0.9% NaCl) was prepared, and a total of 1.5 ml of probiotic solution (equal to 2 x 10^8 CFU/d) was administered totally for 5 days (d) to each rat of probiotic groups by oral gavage. Also, the same volume of serum physiologic (SP) solution was applied totally for 5 d by oral gavage to the other 7 rats of the SP group to make microbiological comparisons with probiotic groups. On the 3rd, 5th, and 9th d of the study timeline, the densities of VRE and probiotic bacteria in fecal samples were measured. All the animals were sacrificed on the 9th d of the study, after 5 d of probiotic treatment.

The study time had been kept longer in previous studies on this topic (9-13) because the antibiotics had been used before VRE inoculation. We did not use antibiotics in our study. In this study, we aimed to investigate the acute histopathological effects of probiotics on the intestinal tract of VRE colonized rats. We did not keep the working time longer as additional confounding factors could be activated as the time went on. Since the animal experiments that were done on this subject were arranged in groups of seven (10,13), also we designed our study in groups of seven.

Animal Sacrifice and Tissue Sampling

General anesthesia was first induced by ether (Galenik, Turkey). A vertical incision was performed and samples were taken from the distal ileum (2 samples) (Di), cecum (1 sample) (Ce), and descending colon (Co), then the animals sacrificed with high-dose ether anesthesia. Samples were stored in neutral formaldehyde solutions and routine follow-up protocol blocks were prepared, and 5 µm-sized paraffin-embedded tissue sections were stained with Hematoxylin and Eosin (Merck, Darmstadt, Germany), followed by they were examined under a light microscope. From each group, 70 samples were obtained and examined.

Histological Examination

The histological evaluation was performed by a histologist who was blinded to the study groups. We described the histological scoring system in Table 1.

Statistical Analysis

Statistical analysis was performed by using SPSS, version 15.0 (IBM SPSS, Chicago, IL). Quantitative data are shown as

Table 1. Grading system in the histopathological examination of the gastrointestinal system of the rats

The lumen of the gastrointestinal system	Score
No debris or epithelial cells	1
Some debris present	2
Intense glandular epithelial cells and debris tissue	3
Intestinal mucosa	
Normal histological structure	1
Epithelial injury present in 25% of the sample	2
Epithelial injury present in 25–50% of the sample	3
Epithelial injury present in more than 50% of the sample	4
Lamina propria	
Normal	1
Mild inflammation and glandular hyperplasia	2
Severe inflammation and edema of the glands	3
Submucosa	
Normal histological structure	1
Mild inflammatory infiltration	2
Severe inflammatory infiltration	3
Injury Grade	
Normal Grade I (mild): < 5 Grade II (moderate): 6–9 Grade III (severe): 10–13	

the mean ± standard deviation (SD) or as medians. Qualitative variables are expressed as absolute and relative frequencies. Categorical variables were compared by using the $\chi 2$ test, and the Student's t-test or Mann–Whitney U test was applied for continuous variables. Loads of probiotics of three probiotic groups and VRE loads in every group at 3-time points (3, 5, and 9 d) were used for the microbiological comparison of the groups. A value of $p< 0.05$ was considered statistically significant.

Results

Comparison of the Probiotic Groups and the SP Group

The median gastrointestinal VRE load was significantly higher at the pre-treatment period (on the 3rd d of the timeline) in the probiotic groups as compared with the SP group ($p = 0.017$). There was no significant difference in the median gastrointestinal VRE load of 3rd d of the treatment (on the 5th d of the timeline) in the probiotic groups versus the SP group ($p = 0.079$) (Table 2). The median gastrointestinal VRE load in the probiotic groups was significantly lower after the 5th d of treatment (on the 9th d of the timeline) as compared with the SP group ($p = 0.021$) (Table 2).

The median gastrointestinal VRE load in the *P. acidilacticii* group was significantly higher on the 3rd d of the timeline as compared with that in the SP group ($p = 0.012$). The median fecal VRE counts in the *S. boulardii* group were significantly lower after the 5th d of treatment (on the 9th d of the timeline) as compared with the SP group ($p = 0.002$). The median fecal VRE counts of the *L. rhamnosus* group were not significantly different as compared with the SP group at any of the time intervals assessed ($p > 0.05$) (Table 2).

Timeline Changes in Fecal VRE Loads in the Probiotic Groups (*L. rhamnosus*, *P. acidilacticii*, *S. boulardii*) and the SP Group

Comparison of the fecal VRE loads of the probiotic and SP groups 3, 5, and 9th d of the timeline revealed a decrease in the

Table 2. Microbiological examination as a VRE count and VRE loads, and histological examination of gastrointestinal system samples from the animals, post-sacrifice. (Min: minimum, Max: maximum)

	Serum physiologic group	Subgroups of probiotics		
		L. rhamnosus **GG**	*P. acidilacticii* **C69**	*S. boulardii*
Microbiological examination Median (Min-Max)				
3rd day	6.0×10^{10} (2.0×10^{10}-7.0×10^{11})	2.0×10^{11} (5.0×10^{10}-4.0×10^{12})	1.0×10^{12} (1.5×10^{11}-5.0×10^{12})	1.0×10^{11} (3.0×10^{10}-7.0×10^{11})
5th day	6.0×10^{10} (2.0×10^{10}-7.0×10^{11})	7.0×10^{10} (3.0×10^{10}-7.0×10^{11})	7.0×10^{10} (8.0×10^{9}-7.0×10^{11})	1.0×10^{10} (7.0×10^{7}-1.0×10^{11})
9th day	4.0×10^{7} (3.0×10^{7}-5.0×10^{7})	5.0×10^{5} (100-7.0×10^{7})	5.0×10^{5} (100-7.0×10^{8})	1000 (100-4.0×10^{5})
Histological examination Number (%)				
Histologically normal	0	15 (21.4)	10 (14.3)	23 (32.9)
Grade I (mild)	0	32 (45.7)	45 (64.3)	47 (67.1)
Grade II (moderate)	19 (27.1)	23 (32.9)	0	0
Grade III (severe)	51 (72.9)	0	15 (21.4)	0

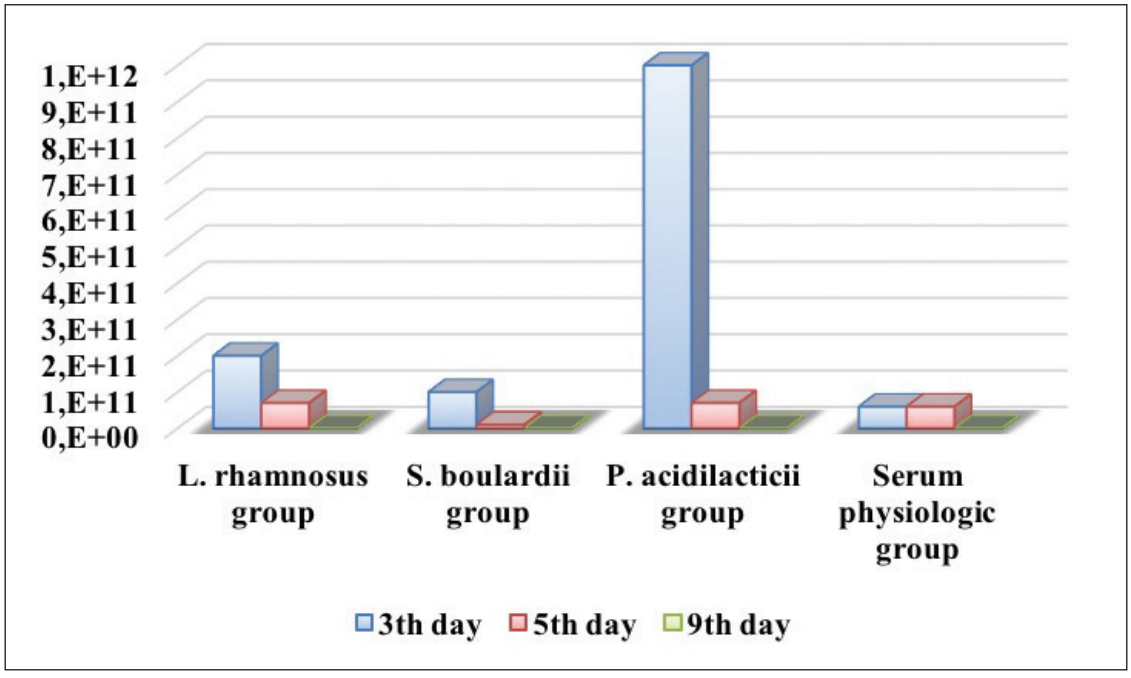

Figure 2. Changes in the fecal VRE load in rats treated with *L. rhamnosus* GG, *S. boulardii*, and *P. acidilacticii* C69 as compared with that of the SP group.

VRE loads in the probiotic groups, but this finding was not statistically significant (p > 0.05). At each of the three intervals assessed, there was no significant difference in the fecal VRE loads between the probiotic groups and the SP group (Figure 2).

Histological Examination of Gastrointestinal Tissue Samples

The ratio of moderate and severe changes in the histological examination of the gastrointestinal systems of the rats was significantly higher in the SP group as compared with the probiotic groups (p= 0.000) (Table 2). Normal viewing histological structures preserved in the *S. boulardii* group but not preserved in the *P. acidilacticii* (p= 0.016) and SP group (p= 0.000). However, no statistical significance was present between the *L. rhamnosus* and *P. acidilacticii* group (p> 0.05) (Table 2). Moreover, in the SP group, all of the 70 samples severely or moderately were affected. In contrast, only 38 of 210 (18.1%) specimens in the probiotic groups were showed moderate to severe damage and moderate to severe damage ratio was significantly higher in the SP group (p = 0.000). In the *S. boulardii* group, moderate to severe damage was not observed in contrast to that observed in the SP, *P. acidilacticii*, and *L. rhamnosus* groups (p= 0.000) (Table 2, Figure 3).

Discussion

In our study, despite the significantly higher VRE load in the probiotic groups at the 3rd d of the study (before applying the probiotics and SP suspensions), the VRE count of the 5th d post-treatment of probiotics was lower than the SP group. Although a completely cleaned culture was not achieved in any of the probiotic groups, some clinical benefits of the probiotics were observed. Despite the failure to eliminate VRE colonization, the protective effect of the probiotics in terms of the gastrointestinal epithelium was well demonstrated. The ratio of severe changes in the epithelium was significantly higher in the SP group as compared with the probiotic groups (p= 0.000).

Most previous studies on the effects of probiotics on VRE had been organized by administering antibiotics before VRE inoculation (9-13). In our study, antibiotics were not administered before VRE inoculation, as we wanted to examine only probiotic effects on the VRE colonization. In a previous study, VRE had been found the dominant bacteria 7 d after the cessation of antibiotics and was cultured up to 2 months after cessation of therapy (14).

In a previous study, Lactobacillus lactis MM19 had been decreased the rate of detectable VanA type VRE, had been pointed to the initiation of probiotics promptly (11). In another study, heat-killed E. faecalis strain EC-12 and undefined Lactobacillus spp. had been achieved a remarkable reduction in the VRE count in chickens (12).

In our research, the median fecal counts for VRE in each of the probiotic groups at the 3-time intervals were significantly lower than those in the SP group. However, as shown by the statistical analysis of changes in VRE rates during the timeline, none of the probiotics were showed superiority in terms of decreasing VRE counts as compared with the SP group in our rat model (p> 0.05).

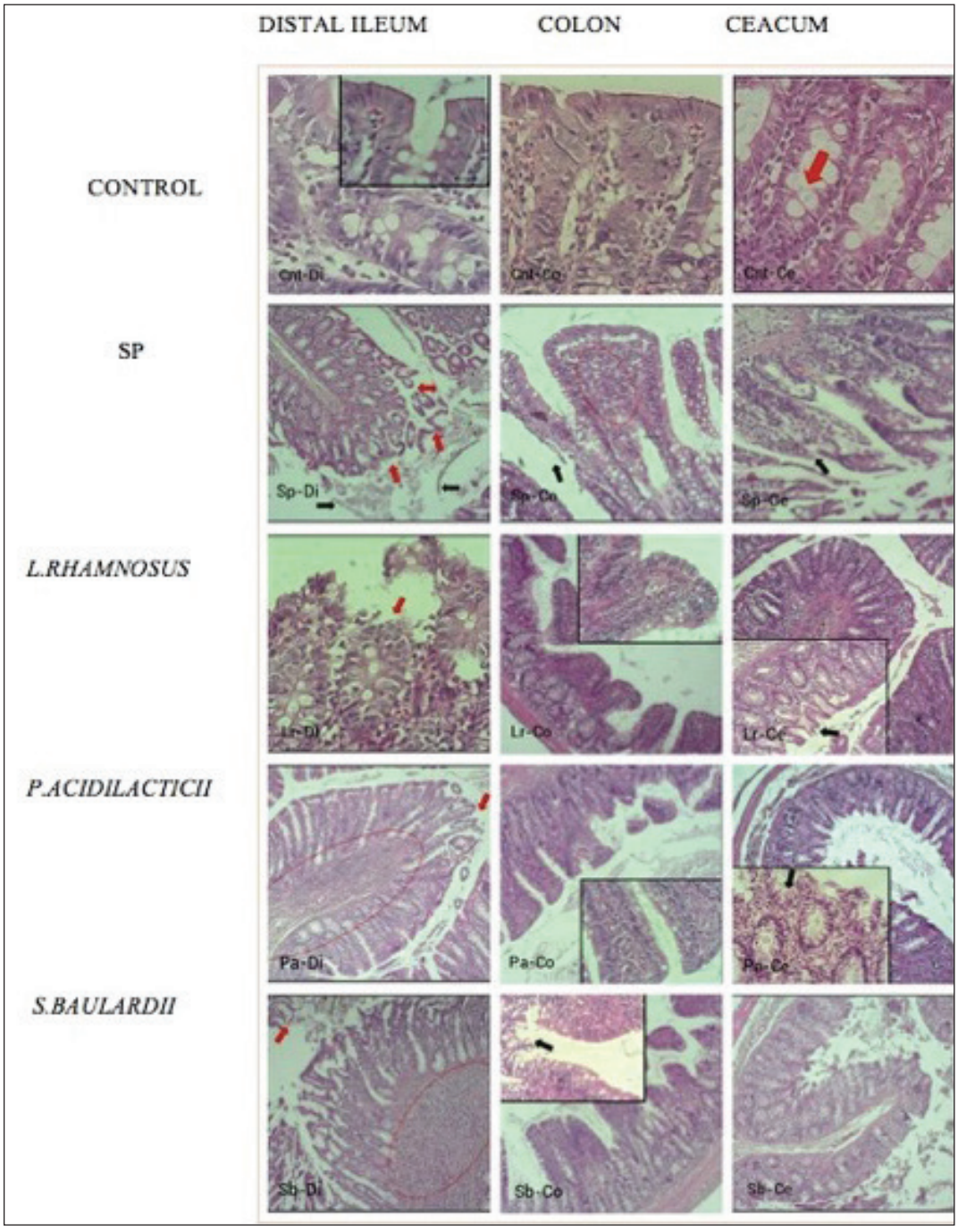

Figure 3. Distal ileum, colon, and cecum images following staining with Hematoxylin and Eosin.

Cnt-Di: Control group, normal appearance of the distal ileum (Di), **SP-Di:** Serum physiologic group, showing the damaged appearance of the distal ileum (red arrows) and spillover of glands and epithelial cells in the lumen. The black arrows denote glandular and epithelial cell secretion and spillover in the lumen. ***L. rhamnosus*-Di:** *L. rhamnosus* GG group, showing superficial damage in the epithelium (red arrow: glandular epithelium damage). ***P. acidilacticii*-Di:** *P. acidilacticii* C69 group, showing superficial damage at the villus and mononuclear inflammatory infiltration in the lamina propria and submucosal areas (dotted circular area), ***S. boulardii*-Di:** *S. boulardii* group, showing mucosal and glandular damage, with inflammatory infiltration (circular area), epithelium, and debris spillover in the lumen (red arrow). **Cnt-Co:** Control group, showing the normal appearance of the colon (Co). **SP-Co:** Serum physiologic group, with local mucosal damage (black arrow) and inflammatory cell infiltration under the epithelium (circular area), ***L. rhamnosus*-Co:** *L. rhamnosus* group GG, showing an image of a normal colon and minimal subepithelial inflammatory infiltration in the thumbnail ***P. acidilacticii*-Co:** *P. acidilacticii* C69 group, showing an image of a normal colon and minimal subepithelial inflammatory infiltration in the thumbnail. ***S. boulardii*-Co:** *S. boulardii* group, showing an image of a normal colon, with minimal epithelial damage in the thumbnail (black arrow). **Cnt-Ce:** Control group, showing a normal epithelium and gland structure of the (red arrow) cecum (Ce) **SP-Ce:** Serum physiologic group, showing heavily damaged areas with glandular epithelial and connective tissue cells spilling into the lumen (black arrow), ***L. rhamnosus*-Ce:** *L. rhamnosus* GG group, with a normal cecum and local superficial epithelial damage seen at larger magnifications in the smaller of the two images (black arrow). ***P. acidilacticii*-Ce:** *P. acidilacticii* C69 group, showing an image of a cecum with general damage and glandular damage in the smaller of the two images (black arrow). ***S. boulardii*-Ce:** *S. boulardii* group, showing dense epithelial damage.

The administration of probiotics had been prevented or eliminated the intestinal VRE colonization by enhancing the density of beneficial intestinal microbiota and modulating immune functions in murine models (13). There are only a few studies on the impact of probiotics on VRE colonization and the findings of these studies were discordant (13-18). In a murine model, the administration of *L. rhamnosus* Lcr 35 had resulted in a decrease in the VRE colonization rate. However, the same study had been failed to observe a similar effect in nine patients given *L. rhamnosus* Lcr 35 (19). One small study on 27 VRE-positive patients, half of whom were treated with yogurt containing *L. rhamnosus*, had been reported the beneficial effects of probiotics on VRE colonization (15). A randomized, single-blinded, placebo-controlled study had been reported that the rate of VRE clearance in colonized children who were treated with *L. rhamnosus* was significantly higher as compared with the untreated group (15). In our research, the study duration was 5 d. In contrast, the duration of probiotic treatment in most previous studies was 3–4 weeks (10,15,17). The relatively long duration of these studies may have affected the results. Also, divergence in the findings of animal studies may be due to variance in microbiota populations, sample sizes, and possibly type of the used probiotic strains.

Several studies had been demonstrated that the anaerobic ability of microbiota might play an important role in the inhibition of VRE (16,19,20). Moreover, reports of in vivo models of gastrointestinal colonization of VRE had been demonstrated that various probiotic food additives would support the functional barrier of the intestine (21-23). Furthermore, as shown by in vitro cell culture models, probiotic strains had been prevented or minimalized the damaging effects of pathogenic microorganisms on epithelial integrity (24). We also demonstrated the protective properties of probiotics against VRE-associated epithelium damage, with *S. boulardii* which was the most effective between the three probiotic strains. The prevention of VRE-induced epithelial damage might play a role in protecting the development of endogenous VRE infections by impeding entry to the circulation from the gastrointestinal tract.

The effects of these three microorganisms can also be demonstrated in humans, but prospective, randomized controlled trials are needed. All three probiotic strains did not rule out VRE colonization. Histopathologically, we found that *S. boulardii* had the best effect on the healing of the intestinal tissue. Prospective randomized controlled trials in humans are needed to see the effects of probiotics on VRE colonization in humans and the clinical effects on VRE colonized patients.

Conclusion

None of the probiotic strains in our research were eliminated the VRE colonization in our rat model, however, they protected against the VRE-associated epithelium damage. *Saccharomyces boulardii* provided the best protection among the three probiotic microorganisms. *Lactobacillus rhamnosus* GG and *P. acidilacticii* were associated with the limited protection of epithelial injury. New strategies for the prevention of VRE-associated epithelial damage or colitis to prevent infection that following the VRE colonization might be focused on probiotics.

Ethics Committe Approval: Our study was started after obtaining permission from Dokuz Eylül University Medical School Hospital Experimental Animal Local Ethics Committee (Protocol Number: 114/2013).

Peer-review: Externally peer-reviewed.

Author Contributions: Concept - ST, ID, OY, GE; Design - ST, ID, OY, GE; Supervision - ST, ID; Resource - ST, OY; Data Collection and/or Processing - ST, ID, OY, GE; Analysis and/or Interpretation - ST, ID, OY, GE; Literature Search - ST, ID, OY; Writing - ST, ID, GE; Critical Review - ST, ID, OY, GE.

Conflict of Interest: The authors declare that there are no conflicts of interest.

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