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IMPACTS OF *SACCHAROMYCES* ON CD20 AND CD68 MARKERS IN DIABETIC RAT SPLEEN IMMUNIZED WITH INFLUENZA VACCINES

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ABSTRACT

A correlation between dysfunctional immune responses and diabetes disease was confirmed years ago. Notably, recommendations for diabetic patients' vaccinations assume great significance because of their susceptibility to infection complications. Interestingly, awareness of the vital role of Saccharomyces cerevisiae (S. cerevisiae) -as a probiotic- in enhancing host immune response has increased lately. Thus, to explore the histological and immunohistochemical effects of Saccharomyces probiotic on the diabetic male Albino rats' spleens after immunization with influenza vaccine, forty rats were randomly divided into four groups including healthy negative controls (C group), positive controls that were injected with 40 mg/kg Streptozotocin (STZ) to induce diabetes disease (G1 group), along with two different rats groups injected with STZ and immunized with either 0.5 ml influenza vaccine only (G2 group) or immunized with influenza vaccines and orally treated with 11.2 mg/kg. wt Saccharomyces probiotics (G3 group). A limited improvement in the histological alterations was observed in the spleen of the G3 group compared to the G2 and G1 groups comparing to those of the C group. The CD68 marker expressions increased in the spleen sections obtained from the G3 group compared to G1, G2, and C group, meanwhile, the expression levels of the CD20 showed an enormous decrease in the spleen sections of the G3, G2, and G1 groups compared to the C group. Consequently, this study reports the withdrawal of the immunomodulatory effect of Saccharomyces probiotics on the immune responses against the influenza vaccine in the spleen.

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Introduction

The spleen is the large secondary lymphoid organ in the body, and it is responsible for the drainage of compounds administered intravenously. Commonly, any foreign antigens are evaluated by the spleen because of its containment of numerous subsets of B and T lymphocytes [1]. The presence of B cells detected due to the expression of CD20+ molecules, where it is a member antigen of B cell [2]. Also, CD20 helps B cells enable an optimal immune response against T-independent antigens specifically [3]. CD68 is a highly expressed glycoprotein molecule on macrophages and other mononuclear phagocytes, for this reason, CD68 consider as a cytochemical marker of macrophages in immunological applications [4]. Phagocytosis increases by the vital role that macrophages play in stimulating the inflammatory response [5]. In 2019, International Diabetes Federation stated that diabetes is almost 463 million individuals worldwide, and the number will rise to 700 million people by 2045. Globally Saudi Arabia is through the top 10 countries with the highest spread of diabetes disease with a ratio of 23.9% [6]. Diabetes is a metabolic disease characterized by abnormal hyperglycemia that is resulted from changes in insulin production, insulin function, or an association of both [7-9]. An increase in glucose in the

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blood is considered the main reason for severe infection in diabetic people, thus impaired immunity (e.g., decline humoral immunity, depression of the antioxidant system, and damage to the neutrophil function) [10].

One of the most significant agents leading to increase acuteness of the influenza virus infection is the impaired immunity [11]. Vaccination is the most effective method to prevent influenza infection [12]. The vaccine response differs from one case to another and it depends mainly on several factors such as the age, health status, and strain of virus used in the vaccine that corresponds to its prevalence in the community. The immune response efficiency of the inactivated vaccine is approximate >60% [13].

There are hundreds of yeast species now identified. One of the most notable yeast species in health and wellness, additionally use as a probiotic is known as *Saccharomyces (S) cerevisiae*, which is also known as brewer's, or baker's yeast [14]. The World Health Organization defines probiotics as microorganisms that when ingested in appropriate quantities can confer benefits for host health [15]. *S. cerevisiae* probiotic has an immunomodulatory activity, where it stimulates the secretion of various cytokines and immunoglobulins due to Toll-like receptor expression. Moreover, *S. cerevisiae* modifies the signaling pathways responsible for the transcriptions of several anti-inflammatory cytokines, leading to a decrease in the inflammation process [16]. So, it is essential to suggest a new era to enhance influenza virus vaccine efficiency in diabetic individuals. The present study evaluated the effects of *S. cerevisiae* probiotic on the spleen immune response of the diabetic rat during immunization with the influenza virus vaccine. The histological and immune histochemical studies -in particular CD20 and CD68 markers- were assessed in different immunized diabetic groups comparable to either untreated diabetic or healthy rat groups.

Materials and Methods

Saccharomyces Cerevisiae

Saccharomyces cerevisiae yeast (Saf-instant yeast made in Turkey) was obtained from the commercial market. The *S. cerevisiae* suitable dose concentration was 11.2 mg/kg/wt dissolved in distilled water [17].

Study Animals and Experimental Design

The experiment was conducted on 40 male albino rats in standard laboratory conditions for eight weeks at the King Fahd Center for Medical Research at King Abdulaziz University in Jeddah, Saudi Arabia. Rats weighed about 200-300 g. The rats were divided into four groups: group C control group; group G1 rats were injected with 40 mg/kg body weight of STZ drug for one time to provoke diabetes disease; group G2 rats treated as similar as the G1 group also injected with one dose (0.5 ml) of influenza vaccine post one week from induction diabetes and left untreated for 14 days; Finally, group G3 included rats treated as similar to the G2 group with a continuous oral administration of *Saccharomyces cerevisiae* three times per week for 15 days before one day from the injection of the influenza vaccine. By day 14, all rat groups were sacrificed, and their spleen tissues were conserved for further histological and immune histochemical studies. Ethical approval for the experiment was obtained from the Scientific Research Ethics Committee at the College of Science at King Abdulaziz University and King Abdulaziz City for Science and Technology (KACST), Jeddah, Saudi Arabia.

Histology of Rats' Spleen

Specimens of the spleen were taken from the control and treated groups. After sampling, they were placed in a solution of 10% paraformaldehyde to be fixed for one hour, to be used in histological and immunochemical studies. The specimens were placed in ascending levels of alcohol for dehydration after washing and cleared in xylene and incorporated into paraffin wax to prepare paraffin blocks. Sections of prepared paraffin blocks were cut at 5µ thickness and then placed in the hematoxylin and eosin stain for histological studies [18].

Immunohistochemistry Methods of CD20 and CD68

The expression of CD20 and CD68 in the spleen was detected by IHC staining with the anti-mouse CD20 and the anti-mouse CD68 (Roche, USA, cat. no. 760-2531, 760-700) respectively. Paraffin blocks were used, and the sections of rats' spleen (4µ thick) were mounted on a glass slide. Slides were heated in an oven for 1 hour at 60 °C then washed with xylene. Spleen sections obtained from different rats' groups were dehydrated in descending grades of ethyl alcohol then washed and rinsed with phosphate-buffered saline (PBS) for 5 min. Sections were immersed in antigen retrieval solution post placing in the microwave at 93°C for 20 minutes. After heat treatment, the slides were cooled to room temperature and rinsed with PBS for 5 min. Anti CD20 or anti CD68 (antibody) was applied and incubated for 16 min at 37°C overnight, followed by rinsing in PBS. Color generator (3,3 di amino benzidine DAB) was added to slides and incubated for 20-30 min then rinsed with PBS and washed in distilled water. Slides were differentiated by staining with Mayer's hematoxylin stain for 4 min then rinsed with distilled water Finally, sections were dehydrated in descending grades of ethyl alcohol than with xylene. The slides were left to dry in the room air for 20 minutes and with (DPX) distance-plasticizer-xylene & coverslip [5].

Statistical Analysis

The statistical analysis of this study was done by used MegaStat software. Significant differences between the different groups were analyzed by one-way ANOVA. Data were normally distributed and are distinct from the mean standard error of the mean (SEM). The differences were considered statistically significant at P <0.05.

Results and Discussion

General Characterization of Rats' Spleen Weight

Diabetic rats treated with influenza vaccines and *Saccharomyces cerevicia*e probiotics group weights exhibited moderate splenomegaly compared to C, G1, and G2 groups; however, this increase is nonsignificant statistically. Meanwhile, the G1 and G2 groups showed significant splenomegaly than those of the untreated healthy group (P= 0.004 and 0.028) respectively, **(Figure 1)**.

Histological Findings in Diabetic Rats' Spleen

Histological findings observed in different rat splenic tissues were subjected to consideration. In the negative control group (C group), Predominant spleen areas were visible including the marginal region, white pulp, and red pulp. Also, the normal central artery was detected **(Figure 2a)**. Meanwhile, the splenic tissues of diabetic rats immunized with influenza vaccine and orally treated with *Saccharomyces cerevisiae* (G3 group) **(Figure 2d)** illustrated several definite changes in its parts. The white pulp lymphocytes characterized by a decrease in the depletion areas compared to those of the untreated diabetic rat (G1) **(Figure 2b)** and diabetic rat immunized with influenza vaccine (G2) **(Figure 2c)** groups, and it appeared more similar with those of the untreated control group **(Figure 2a)**. In term of mononuclear cells proliferation, and lymphoid tissue hypertrophy, which led to a decrease in the number of lymph follicles and a loss of overall architecture of the marginal zones verified in the G3 spleen sections compared to G1 **(Figure 2b)** and G2 **(Figure 2c)** groups. The thickness of the central artery of the spleen cells of the G3 group **(Figure 2d)** seemed more similar to the G2 group **(Figure 2c)**.

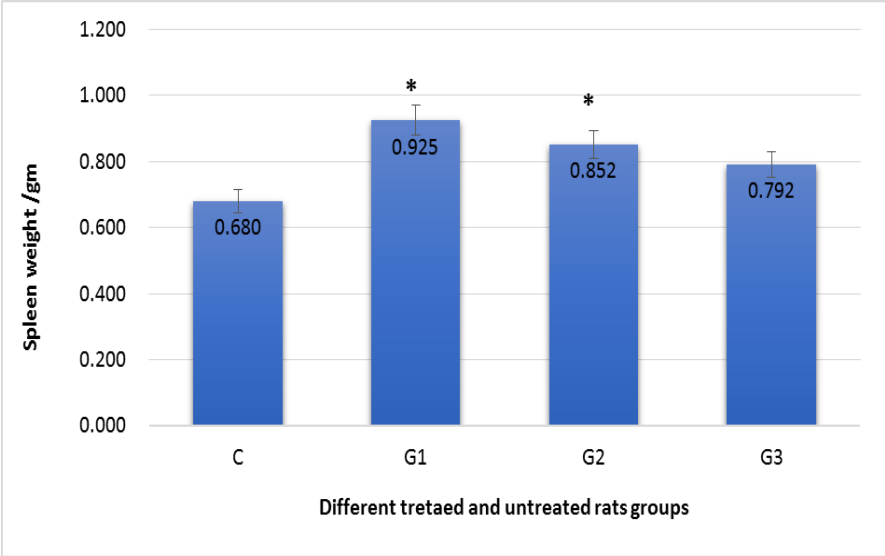
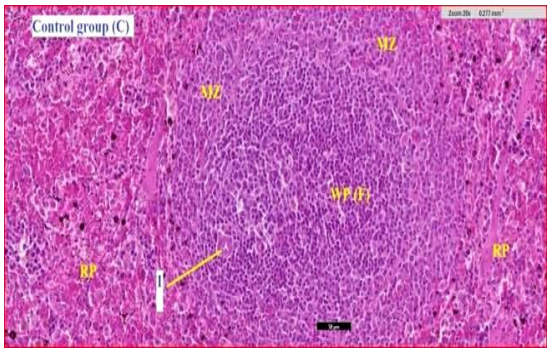


Figure 1. Diagram shows the spleen weight obtained from different rat groups, where C represents serum from the untreated healthy group of rats and (G1) represents serum of diabetic rats. (G2) represented serum of diabetic rats immunized with influenza virus vaccine, and (G3) represent serum of diabetic rats treated with both influenza virus vaccine and *Saccharomyces cerevisiae* probiotics. (*) Significant at p<0.05 as determined by analysis of variance, the comparison was performed using the One-factor ANOVA test. (*) Comparison between diabetic groups and untreated healthy groups. Every point represented the mean value of six separate tests. The vertical bars denote the 5% percentage around the mean.



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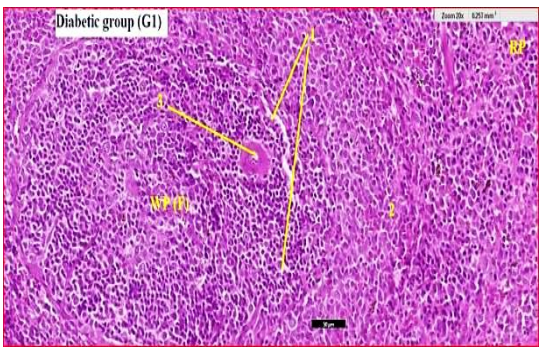

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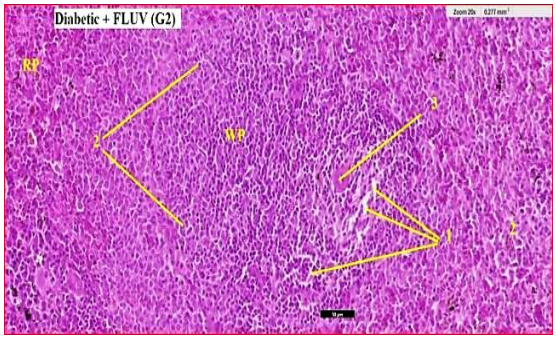

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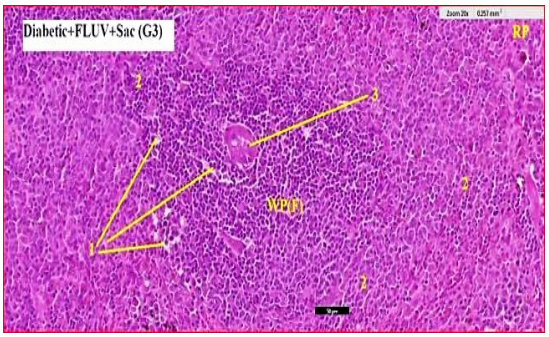


d)

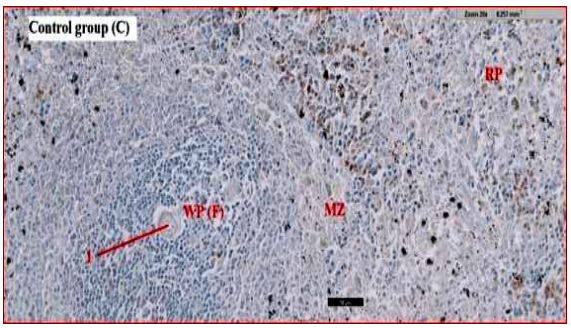
Figure 2. Histological findings of spleens of treated and untreated rat groups. (a) untreated control (C group), (b) diabetic rat (G1 group), (c) diabetic rats after immunization with influenzas vaccine (G2 group). (d) diabetic rats after immunization of influenzas vaccine and administration of *Saccharomyces cerevisiae* probiotic (G3 group). showed distinct red pulps (RP) and a white pulp follicle (WP(F)) surrounded by a marginal zone (MZ). (1) represented the depletion area in the white pulp region. (2) showed the activation of white pulp (WP) and red pulp lymphocyte (RP), and the disappearance of the marginal zone. (3) represented thicken in the wall of the central artery. 20X, all sections were stained with (H&E).

Estimations of CD20 Levels in the Spleens of Different Rat Groups

Rats' spleen sections were stained with an anti CD20 marker to discern the B cells, that interpreted as brown color dotes. Each B cell marker in the spleens of the four rat groups -C, G1, G2, and G3- were evaluated individually. As shown in **Figures 3g, 3h, and 3i**, the immunohistochemistry positive reactivity pattern of the CD20 in the spleen section of the G3 group confirmed a nonsignificant decrease compared to those detected in the C group that verified high levels of staining intensity **(Figures 3a, 3b, 3i)**, while it is increased insignificantly than those of the G1 group **(Figures 3c, 3d, 3i)**. Also, the G3 spleen sections verified an extremely significant decrease in the CD20 expression compared to those of the G2 group (P = 0.001) **(Figures 3e, 3f, 3i)**.

Estimations of CD68 Levels in the Spleens of Different Rat Groups

Anti CD68 -brown color dotes- was detected to realize monocytes and macrophages cells in the spleen section of the current treated and untreated rat groups. Macrophage and monocyte markers in the spleens of the four rats' groups were evaluated individually. Regarding untreated control spleen sections, CD68 resided moderately in the red pulp region, while both white pulp and the marginal zone showed a rare of them **(Figures 4a, 4b, 4i)**. As shown in **Figures 4g, 4h, and 4i**, the CD68 expression increased significantly in the spleen cells regions -particularly the red pulp region- of the G3 group compared to those of G1 **(Figures 4c, 4d, 4i)**, G2 **(Figures 4e, 4f, 4i)** and C groups (P= 0.002, 0.0113, and 0.0008) respectively.



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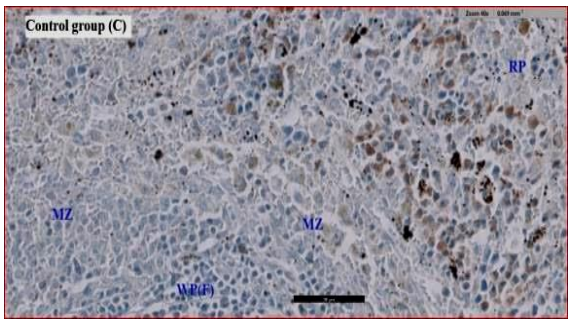

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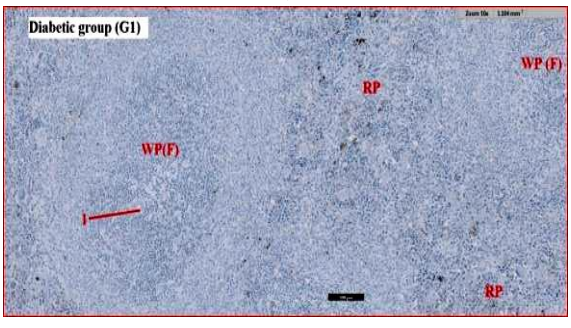

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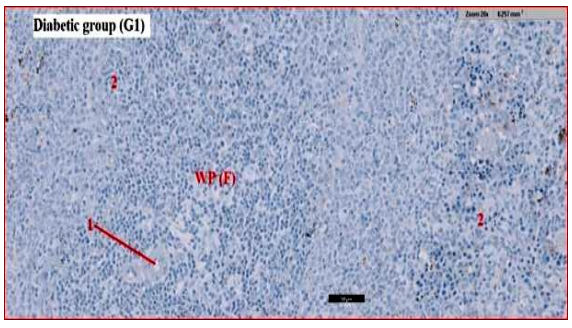

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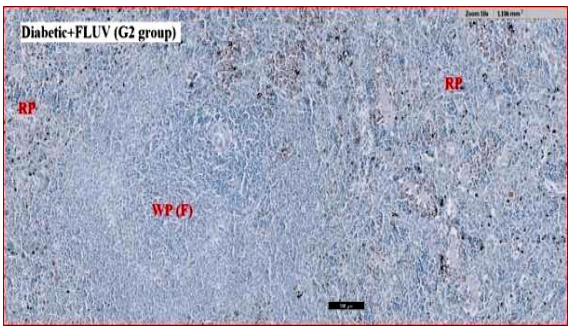

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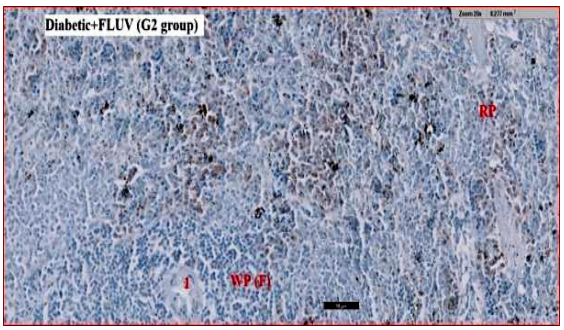

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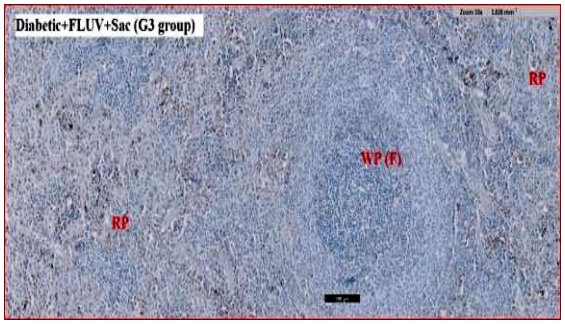

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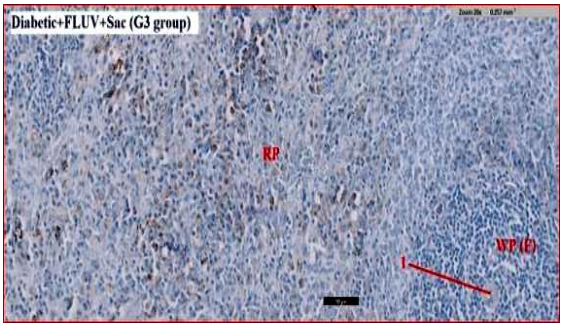

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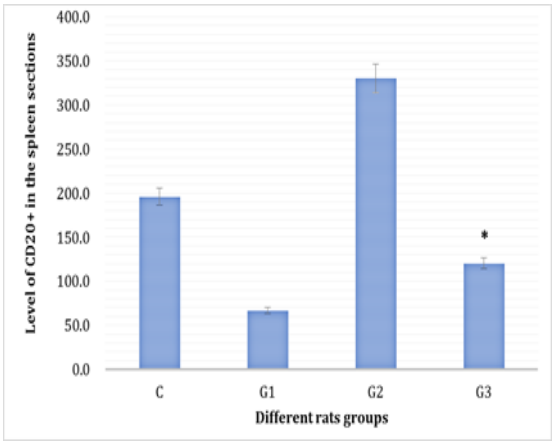
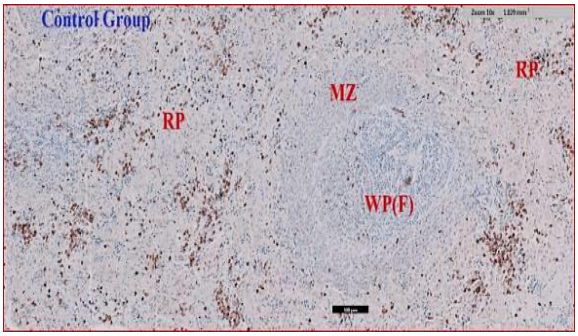


i)

Figure 3. Sections of rat spleens stained with CD20 marker to distinguish B lymphocytes. CD20 expressions -brown color dotes- was observed in the marginal zone (MZ), the red pulp (RP), and the central artery (1) in the untreated control (C group) (a, b), diabetic rat (G1 group) (c, d), diabetic rats after immunization with influenza vaccine (G2 group) (e, f), and diabetic rats after immunization of influenza vaccine and administration of *Saccharomyces cerevisiae* probiotic (G3 group) (g, h). The white pulp follicles (WP(F)) in all diabetic groups seemed virtually free from the CD20. CD20+ cells vanished in the MZ and RP of the diabetic group (G1), but they increased in the RP region of the G2 and G3 sections groups. (i) The values shown are the mean count of the CD 20+ cells in the spleen sections. (*) Significant at $p<0.05$ as determined by analysis of variance, the comparison was performed using the One-factor ANOVA test. (*) Comparison between G3 and G2 groups. Every point represented the mean value of six separate tests. The vertical bars denote the 5% percentage around the mean.



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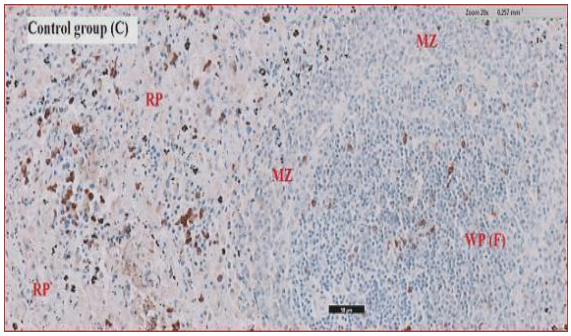

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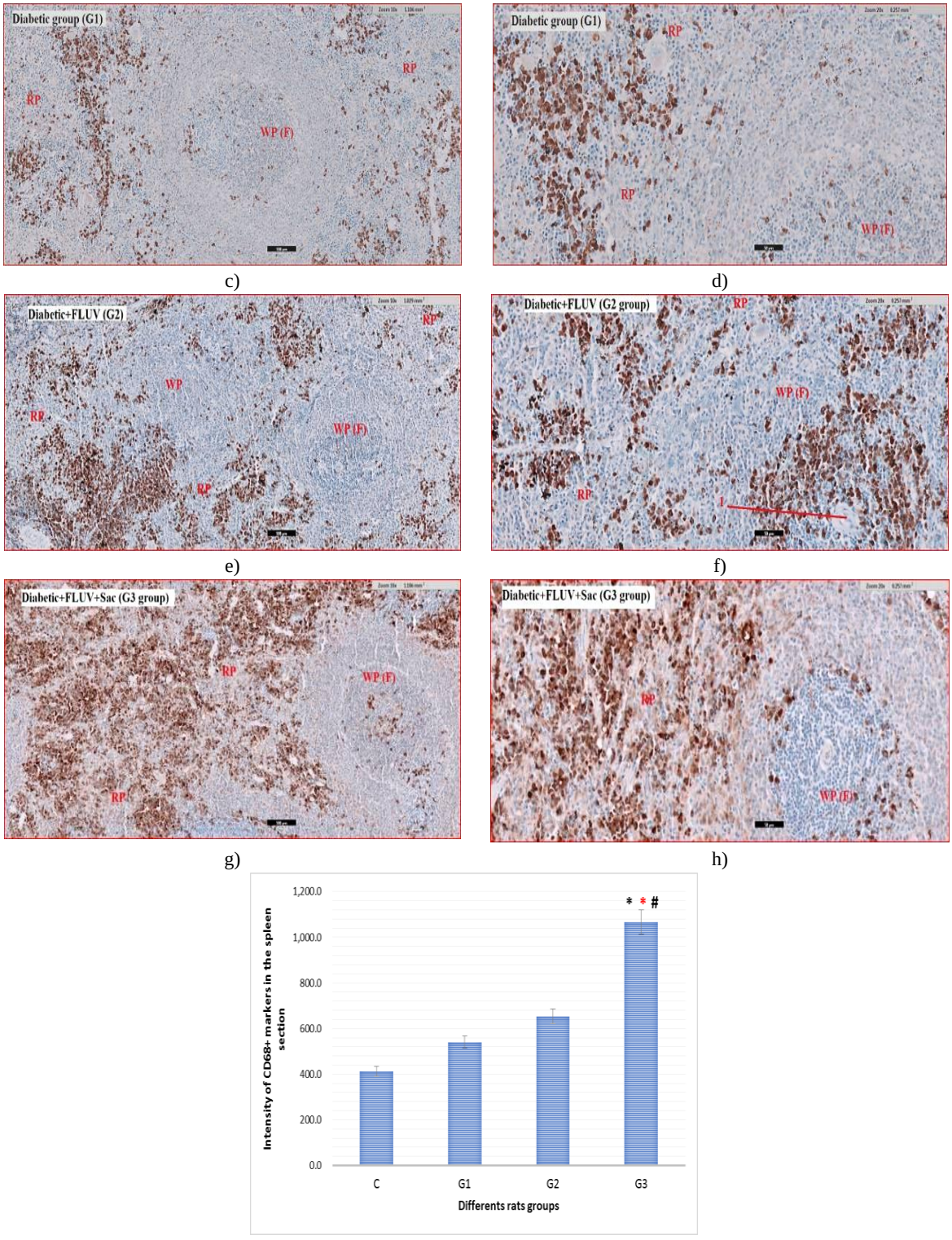

Figure 4. Sections of rat spleen stained with CD68 cells to distinguish macrophages and monocytes cells. CD68 expressions -brown dotes- increased in the red pulp (RP) region, while it decreased in the white pulp follicle (WP(F)) and the marginal zone (MZ) in the untreated control (C group) (a, b). Diabetic rat (G1 group) (c, d), diabetic rats after immunization with influenza vaccine (G2 group) (e, f), and diabetic rats after immunization of influenza vaccine and administration of *Saccharomyces cerevisiae* probiotic (G3 group) (g, h) showed a decrease in the expressions of the CD68 in the WP(F) and MZ regions. While the RP regions of all diabetics' groups showed an intensive increase in the CD68+ cells. (i) The values shown are the mean count of the CD68+ cells in the spleen sections. (*) Significant at $p<0.05$ as determined by analysis of variance, the comparison was performed using the One-factor ANOVA test. (*) Comparison between G3 and C groups, (*) comparison between G3 and G1 groups. (#) Comparison between G3 and G2 groups. Every point represented the mean value of six separate tests. The vertical bars denote the 5% percentage around the mean.

Manifold reasons have interfered with the immunity comptonization of diabetic patients, which lead to increasing the incidence of infection and disease complications in diabetic than healthy people [19]. Diabetic people are six times more likely to be hospitalized during an influenza epidemic than healthy ones, with the mortality varying between 5% and 15% from total mortality 10,000–30,000 annually due to influenza epidemics [20]. Vaccination is an effective process that can provide protection against diseases-at least partially protection-, reduce disease-associated complications and decrease hospital admissions [21]. Awareness of *Saccharomyces cerevisiae's* vital role as probiotics in improving immune response has increased lately [22, 23]. *Saccharomyces* has the potential to stimulate both innate and adaptive response of the host cell immunity [24], such as stimulation of monocytes, the phagocytic function of macrophages, neutrophils, and natural killer cells [25]. These drove to the question of whether *Saccharomyces cerevisiae's* probiotics would affect CD20 and CD68 markers in the spleen of diabetic rats' post-immunization with the influenza vaccine. Also, did diabetes disease associate with any spleen histological alterations of rats before and after influenza immunization.

The spleen is the greater lymphoid organ in the body that prosperous with different immune cells and stimulates immune responses against pathogens. Its functions are related to the systemic circulation system and it lacks lymphatic vessels [26]. Our results agree with studies that confirmed the significant alterations in the splenic tissue weight of diabetic rat groups [27]. Both current diabetic (G1) and diabetic rats immunized with the influenza vaccine (G2) group showed expansion of the red and white pulp, indicating that there is an enlarged spleen compared to the control rat group at day 21 [28]. In the present study, the spleen weight of diabetic rats immunized with influenza vaccines and fed *Saccharomyces* probiotics (G3) showed a decrease in the spleen enlargement compared to those of G1 and G2 groups; however, this decrease was nonsignificant and seemed more similar to the untreated control group. This reduction in the G3 spleen enlargement may be due to abatement of white and red pulps cells [28], and *Saccharomyces* probiotic could restore spleen weight beyond normal status.

In the current study, microscopic examinations of the splenic sections of untreated rats consist of two morphologically distinct regions, the red pulp and the white pulp [29]. The histological red pulp consisted of erythrocytes, granulocytes, and a network of splenic cords and venous sinuses within which mononuclear cells are spread [30]. The splenic cords are connected with hematopoietic and lymphocytes cells [26]. The marginal zone, follicles, and periarteriolar lymphoid sheath are the main three compositions of the spleen white pulp region [30]. In the current histological alterations, the diabetic rat spleen treated with *Saccharomyces* probiotics and immunized with influenza vaccine (G3) showed a reduction in the white pulp lymphocytes associated with decreasing in the depletion areas. Also, G3 spleen sections still showed a loss of overall architecture of the marginal zones compared to those of G1and G2 groups. Although the current spleen of the G3 group showed a noticeable improvement on the histological level, the effects of diabetes disease on the spleen tissues are still predominant, that characterized by increasing the cellularity in the white pulp, and changes in the ratio between the white and red pulps [18] as the current diabetic rats' groups compared to those of the untreated control rat.

CD20 -the specific antigen for B cell lymphocytes- is achieved on most of the B cell development stages since the pre-B cell phase and until prior differentiation to plasma cell [31, 32]. Although the biological activity of CD20 has not been fully elucidated, it acts as iron and Ca2+ channels [33]. In the current study, CD20 expression levels in the spleen of the G3 and G2 groups were slightly lower than the untreated control group, but it elevated in comparison with those of the G1 group. B cell atrophy correlated directly to hyperglycemia in mice [34, 35]. B cell deficiency varied according to the immune tissues such as peripheral blood, lymph nodes, and bone marrow, and which was relatively low in the spleen [36]. The current results verified no difference in CD20 expression levels in the G3 and G2 spleen, referring that immunization with influenza vaccine gradual restoration of the B lymphocytes level following its deficiency due to the diabetes disease [18, 36] Notably, the current results illustrated the low impacts of *Saccharomyces* probiotics on the expression levels of the CD20 in the spleen of the immunized diabetic rat group.

CD68 is a unique member of the scavenger receptors related to class D [37]. It is commonly used as a macrophage marker [4]. In addition to macrophages, several immune cells can express this molecule such as basophils, neutrophils, mast cells, and dendritic cells [38], which is detected by immunohistochemistry techniques, where it appears in a finely granular positivity or dot-like cytoplasmic [37]. In the present study, the spleen of G3 diabetic rats verified an extensive infiltration of the CD68 markers -particularly in the red pulps regions- compared to those of the untreated control, the untreated diabetic, and diabetic rats that immunized with influenza vaccines groups. Generally, several types of vaccines included cancer vaccines capable of stimulating the macrophages' responses [39, 40]. Diabetes disease is a proinflammatory disease [40]. Lately, Eguchi *et al.* [41] appeared expansion in a subset of M1-like macrophages which expressing Ly6c within the islet of the diabetic mouse**.** This increase in macrophages is interesting, T2D is associated with the stimulation of proinflammatory cytokines in the systemic compartment, thus causing chronic, low-grade inflammation [42]. Our results were clear proof that the *Saccharomyces* probiotics succeeded in activating the local macrophages cells in the spleen of the diabetic rat during the immunization of the influenza vaccine. So, proinflammatory cytokine secretion (IL-6, IL-12, TNF-α,) and ROS generation may be affected by increasing the infiltration of macrophages [43].

Conclusion

It was illustrated the limitaaation effects of *Saccharomyces* probiotics on the immune responses against influenza vaccine in the spleen of diabetic rat. *Saccharomyces* probiotics improved the histological modifications in the spleen of diabetic rats compared to those of diabetic untreated control or diabetic immunized with influenza vaccine groups; however, this

histological improvement wasn't enough to resemble those of the healthy untreated control group. Also, *Saccharomyces* probiotics increased the expression of the CD68 in the spleen of diabetic rats compared to those of diabetic untreated control, diabetic rat immunized with influenza vaccine, and the uncontrol groups, suggesting the capability of this current probiotic to increase the proinflammatory cytokines. Meanwhile, *Saccharomyces* probiotics may affect the generation of antibodies against the influenza vaccine due to the insufficiency of the B cell lymphocytes in the diabetic rats' spleens, where a definite inhibition in the expression level of CD20 levels was observed in all the spleens of the diabetic rat groups in comparison with the healthy control group.

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Conflict of interest: None

Financial support: None

Ethics statement: The experimental protocol was established, according to the ethical guidelines and was approved by the Institutional Animal Care and Use Committee (IACUC) of King Abdulaziz University and King Fahad for the Medical research (IACUC). The animals were obtained from the King Fahad for the Medical research. The detail of mice euthanasia and scarification methods is following the IACCU guideline. This research is not an application for clinical research, an institutional review board (IRB) is not applicable.

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